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Diagnosis and Reversal of Multidrug Resistance in Paediatric Cancers

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INTRODUCTION

CANCER IS the second most common cause of death in children [1]. Chemotherapy is pivotal for the cure of childhood cancers since surgery or radiation alone rarely achieves cures [2]. Drug resistance, particularly multidrug resistance, is the most crucial of several obstacles to cure (tumour biology, pharmacokinetic and host factors, drug 'sanctuaries') [3]. P-glycoprotein (Pgp) was the first important multidrug resistance protein described [4]. Clinical relevance is undefined for the multidrug resistance protein (MRP), lung resistance protein (LRP), topoisomerase II, or glutathione enzymes, that may also confer multidrug resistance [5–8]. Pgp confers broad-spectrum resistance to anticancer antibiotics, vinca alkaloids, epipodophyllotoxins and anthracyclines that are important clinically [9]. We will focus this review on the clinical diagnosis and reversal of multidrug resistance in paediatric cancers, since Pgp is of proven relevance in human malignancies [10–26].

P-GLYCOPROTEIN IN NORMAL TISSUES

The 170-kDa membrane-bound Pgp is a well characterised drug efflux pump [27–29] that is widely expressed in normal kidney, liver, adrenal cortex, intestine, blood–brain and blood–testicular barriers, and peripheral blood and bone marrow haematopoietic cells [30–38]. Studies in mice homozygous for the disrupted *mdr1a* and *mdr2* genes (homologues of human *MDR1* and *MDR3*) suggest that the class I and III Pgp isoforms encoded by the *MDR1* and *MDR3* genes fulfill crucial physiological functions. For example, the class I protein protects against toxins at the blood–brain barrier, and the class III protein regulates phosphatidylcholine secretion into bile [39–42].

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P-GLYCOPROTEIN IN HUMAN CANCERS

There are two human Pgps. The class I protein encoded by the overexpressed, rather than by the amplified, *MDR1* gene, confers multidrug resistance in human cancers. The class III protein is rare except in certain B-cell malignancies in which its role is unknown [43]. Some cancers (renal, colon) are constitutively highly positive for Pgp [44, 45]. Other cancers (lung, myeloma, breast, ovary, lymphoma, acute myeloid leukaemia) are more frequently positive for Pgp at relapse (30–80%) than at diagnosis [10, 13, 17–26, 46–51]. Less information on multidrug resistance is available in childhood cancers, and data derived from studying adult malignancies are not directly transposable to paediatric cancers because of major differences in tumour biology and response to chemotherapy. We previously detected low to moderate levels of Pgp, and small numbers of positive tumour cells in 10–30% rhabdomyosarcoma, neuroblastoma and retinoblastoma at diagnosis, and higher expression of the protein and larger numbers of positive tumour cells in nearly all cases at relapse [14–16, 52, 53]. Others have concurred with our findings of neuroblastoma [11, 12].

IMPORTANCE OF CLINICAL CORRELATIVE STUDIES

Correlative studies defining the clinical relevance of Pgp to therapeutic outcomes are essential to provide a rationale for conducting trials to block this protein, thereby improving the efficacy of chemotherapy [10, 13, 54–64]. It is crucial to initiate potentially toxic trials only for tumour types in which Pgp is clearly rate-limiting for response to chemotherapy. To prove that Pgp limits the response to therapy is not easy, since increased Pgp must be found in unresponsive patients, but absent in cured patients; prospective testing should predict the outcome accurately; and Pgp-blockers must salvage resistant

patients. To date, the best evidence for relevance of Pgp as a cause of clinical multidrug resistance occurs in studies of myeloma, lymphoma and acute myeloid leukaemia of adults, and rhabdomyosarcoma and neuroblastoma of children [10–22, 24–26, 49, 51]. In these studies, increased expression of Pgp correlated strongly with poor prognosis, and undetectable expression of the protein correlated with lasting remission.

REASONS FOR DISPARATE RESULTS OF CLINICAL CORRELATIVE STUDIES

However, other correlative studies reported disparate frequencies of increased Pgp in neuroblastoma, lymphoma, acute myeloid leukaemia and Ewing's sarcoma, and disputed the relevance of this protein to clinical multidrug resistance [65–70]. Such discordant results may arise because of differences in evaluating *MDR1* mRNA versus the protein versus drug efflux function. Discrepant results may also be due to differences in studying pooled cells versus single cells. Individual techniques, antibodies and molecular probes for Pgp may affect sensitivity and specificity of assays. Differences in study methodology and criteria for interpretation of results may generate inconsistent conclusions. For example, a contentious issue in multidrug resistance literature is how to define 'negative' and 'positive' groups. According to one definition, the 'negative' group has no detectable Pgp, and the 'positive' group, any degree of positivity. Since low Pgp expression presumably also contributes to failure of therapy, this definition appears most valid. An alternative definition of the 'negative' group includes both detectable Pgp and low levels of positivity, and only refers to high-expression as 'positive'. This definition may underestimate the clinical importance of low levels of multidrug resistance. Some studies have already shown that a few detectable positive tumour cells or low levels of Pgp correlate with poor prognosis [14–16].

POOLED-CELL VERSUS SINGLE-CELL ASSAYS

Pgp assays vary significantly in sensitivity and specificity. Pooled-cell assays of *MDR1* mRNA (slot blot, Northern blot, RNase protection, reverse transcriptase polymerase chain reaction RT–PCR), the protein (Western blot, flow cytometry), and drug efflux function (fluorescent dye or drug efflux on flow cytometry) are quantitative and generally sensitive [71–80]. However, they cannot distinguish between Pgp expressed in tumour cells and the protein present in normal cells [30–38]. Pooled-cell assays also fail to differentiate Pgp in the plasma membrane from cytoplasmic proteins crossreactive with anti-Pgp antibodies, such as a 200-kDa protein migrating with myosin, or the mitochondria enzyme pyruvate carboxylase [81, 82]. Pooled-cell assays do not assess the heterogeneity of Pgp expression in clinical samples, and potentially may miss small numbers of positive cells in predominantly negative tumour samples [14–16]. Except for RT–PCR analysis, pooled-cell assays generally require relatively large fresh or frozen tumour samples. Conversely, single-cell assays of *MDR1* mRNA (mRNA *in situ* hybridisation) or the protein (immunohistochemistry) allow evaluation of the heterogeneity of Pgp expression in clinical samples [14–16, 52, 53, 67]. Morphological examination of single cells is essential for distinguishing tumour cells from normal cells, both of which may express Pgp, and for localising Pgp in the plasma membrane and Golgi region of tumour cells [83]. Small sequential tumour biopsies and archival material are evaluable. However, single-cell assays, except for mRNA *in situ* hybridisation, are qualitative or semiquantitative and only allow objective and quantitative measurement of Pgp when combined with computerised image analysis [84].

MDR1 *RNA assays*

Clinical correlative studies of adult acute myeloid leukaemia, colorectal carcinoma and neuroblastoma measured *MDR1* mRNA in pooled cells by slot blot, Northern blot or RNase protection assays [17–19, 49, 51, 66, 67, 85]. Most clinical studies were descriptive. Some studies showed a significant correlation between *MDR1* expression and the initial response to chemotherapy. Only a few studies correlated *MDR1* expression with long-term outcome of therapy. Although *MDR1* mRNA assays are sensitive, contamination of bone marrow and tumour samples by Pgp-expressing normal haematopoietic or stroma cells presents a significant problem for the interpretation of results [30–38]. This is particularly true for highly sensitive RT–PCR assays. Their usefulness as a clinical diagnostic tool for Pgp requires further evaluation. Very few studies showed a significant correlation between *MDR1* mRNA on RT–PCR and response to chemotherapy, and none to long-term therapeutic outcome [86–88]. *MDR1* mRNA *in situ* hybridisation is technically demanding but may resolve interpretation problems due to contamination from Pgp-expressing normal cells [67]. This assay also requires evaluation in clinical correlative studies. There are no studies showing a significant correlation between *MDR1* mRNA *in situ* hybridisation and response to chemotherapy.

Western blot analysis

The limitations described for other pooled-cell assays apply to Western blot analysis. Western blot cannot distinguish between Pgp-expressing tumour cells and normal cells. Furthermore, even with enhanced chemiluminesence detection systems [89], Western blot assays currently lack sensitivity for detection of low or heterogeneous Pgp expression in clinical samples [76, 90]. Clinical correlative studies of Pgp rarely employ Western blot analysis [46, 75, 91]. There are no clinical studies showing a significant correlation between Pgp on Western blot and response to chemotherapy. Western blot analysis remains most useful for quantifying Pgp contents of control tumour cell lines to standardise *MDR1* assays.

Immunohistochemical assays

Immunohistochemical assays for Pgp are particularly useful for conducting retrospective clinical correlative studies. Diseases studied so far included acute myeloid and lymphoblastic leukaemia, lymphoma, myeloma, breast carcinoma and rhabdomyosarcoma, neuroblastoma and retinoblastoma [10, 13–16, 22, 23, 25, 47, 48, 52]. These studies are vital for their testing of sequential clinical samples before and after chemotherapy. Prolonged follow-up of patients ensured that outcomes are durable. The results of most clinical correlative studies significantly support the relevance of Pgp to response to chemotherapy and long-term outcome. However, assays must be sensitive enough to detect low levels of clinical multidrug resistance, since low expression of Pgp and a few positive cells at diagnosis may contribute significantly to treatment outcome [15, 16].

Flow cytometry

Studies of Pgp in leukaemia and 'liquid tumours' often employ flow cytometry [92, 93]. Most clinical studies have

been descriptive; very few showed a significant correlation between Pgp expression and initial and long-term response to chemotherapy [20, 24]. The same limitations described for other pooled-cell assays apply to flow cytometry. Flow cytometry cannot distinguish between Pgp-positive tumour cells and normal cells. Even simultaneous assessment of other immunological markers may not conclusively differentiate tumour cells from normal cells. Furthermore, some investigators have reported a positive association between Pgp and other prognostic markers. For example, both CD34 and Pgp expression have frequently occurred in acute myeloid leukaemia blasts, and both correlated with adverse outcomes [20]. Yet, normal haematopoietic stem cells can also express CD34, as well as Pgp. Therefore, it is vital to stratify statistical analyses of outcome by concurrently expressed prognostic markers. However, very few studies employ such stratification [14–16, 20].

Pgp functional assays

These assays measure the efflux of fluorescent anticancer drugs (daunorubicin, doxorubicin) or dyes (rhodamine 123, DODC iodide) by multidrug-resistant tumour cells [34, 35]. Verapamil or cyclosporin blockade of drug and dye efflux confirmed the Pgp pump function [77–80]. Only a few clinical studies have shown a significant correlation between Pgp function and initial or long-term response to chemotherapy [26, 94]. Functional assays for Pgp require viable fresh or frozen malignant cells and are usually only applicable to leukaemias and 'liquid tumours'. Present functional assays are only moderately sensitive, and may miss small numbers of tumour cells with low expression of Pgp. Furthermore, drug efflux function does not necessarily correspond with Pgp measurement [95, 96]. This may be due to the inability of functional assays to distinguish between Pgp efflux pump function of tumour cells and normal cells. Uncharacterised ABC transporters may also alter drug efflux function [97].

PREREQUISITES FOR CLINICAL CORRELATIVE STUDIES

To answer the question of whether Pgp causes clinically relevant multidrug resistance, we require: (a) sensitive, unequivocal assays for detection of Pgp; (b) strict methodology for correlative studies; (c) rigorous interpretation of samples.

Sensitive, unequivocal Pgp assays

Evaluating the expression of Pgp by multiple methods is ideal but difficult because of constraints of sample size [98]. Studies should employ two corroborative assays when possible. Assays should be sensitive enough to detect low levels of Pgp and small numbers of positive tumour cells before treatment. Establishing the specificity of assays is important. For example, using two monoclonal antibodies directed against different epitopes on Pgp helps confirm specificity of immunohistochemical assays [99–104]. Epitope-specific peptides that inhibit antibody binding to Pgp also confirm specificity [101]. Negative controls should consist of cell lines expressing no detectable Pgp, as well as isotype matches for the anti-Pgp antibodies. Positive controls should consist of cell lines with different levels of Pgp. Standardising the Pgp and *MDR1* mRNA contents of positive controls by Western or Northern blot analysis is essential. For this reason, normal adrenal cortex and kidney tissues are unsuitable as positive controls because their Pgp expression is very variable. Furthermore, a low-resistant positive control cell line is crucial for ensuring that the assay is sensitive enough to detect potentially low levels of tumour Pgp. Standardising the antibodies and molecular probes for Pgp assays is equally important.

Strict correlative study methodology

Rigorous methodology is essential for conducting correlative studies. Studies of sequential tumour samples before and after treatment yield useful natural history data on clinical multidrug resistance. However, it is the Pgp expression at diagnosis, rather than at relapse, that provides the most valid correlation with outcome, by avoiding the confounding effect of relapse. Studying consecutive patients with the same diagnosis avoids selection bias. Prolonged follow-up (>5 years) ensures that outcome of patients is stable. Retrospective studies are therefore necessary for their prolonged follow-up, but prospective studies are desirable, despite shorter follow-up, for confirming retrospective findings. Having a sufficient number of patients studied allows stratifying statistical analysis of outcome by their different prognostic factors and therapies [105, 106]. Some prognostic factors, such as stage, affect outcome profoundly.

Rigorous interpretation of clinical samples

For immunohistochemical studies, observers should be 'blinded' to the identity of samples and Pgp scores of each other. Rigorous criteria are essential for interpretation of results. It is necessary to score the entire section, and for large samples, score multiple sections. Positive tumour cells should have distinct plasma membrane and/or Golgi staining for Pgp. We advocate disregarding cytoplasmic staining without plasma membrane staining as possibly due to non-specific proteins crossreacting with anti-Pgp antibody [81–83]. We should see Pgp staining in tumour cells rather than only in normal stroma or haematopoietic cells. We advocate scoring as 'negative' only those samples with no positive tumour cells. Since even low Pgp expression may be clinically relevant, we advocate scoring samples with any degree of expression or any number of positive tumour cells as 'positive'. The consensus score of observers should determine the final result. Should there be discordance, we advocate reporting the statistical significance derived from correlating each observer's set of Pgp scores with outcome. Such rigorous criteria for interpretation must apply to all Pgp assays.

CLINICAL CORRELATIVE STUDIES IN PAEDIATRIC CANCERS

Few paediatric cancer studies have attempted to correlate Pgp expression with therapeutic outcomes. This part of the review will focus on studies of neuroblastoma, rhabdomyosarcoma and osteosarcoma.

P-GLYCOPROTEIN EXPRESSION IN NEUROBLASTOMA

Neuroblastoma is the most common cause of paediatric cancer fatalities. Age and stage broadly divide tumours into two prognosis and therapy groups. Early stage (I–II) tumours curable by surgery or disseminated tumours in infants (IVS) that require minimal therapy are favourable. Advanced stage (III–IV) tumours in older children (≥1 year of age) are unfavourable. Their cure rates are 13–36% despite chemotherapy, surgery, radiation and bone marrow transplantation

[107–109]. Why age is critical for outcome is unknown. Traditional prognostic factors besides age and stage include serum ferritin, Shimada histological classification, and urinary vanillylmandelic acid:homovanillic acid (VMA:HVA) ratio [110–112]. *MYCN* oncogene amplification correlates strongly with adverse outcome [113]. Chromosome 1p loss and ploidy in infants may also have prognostic significance [114, 115]. The clinical relevance remains undefined for a large number of other prognostic factors, including ganglioside GD2, neuropeptide Y, neuron-specific enolase, bcl-2 apoptosis-suppressing protein, *trk* oncogene, and nm23 metastasis-related protein [116–120]. We advocate that any correlation of Pgp expression with outcome should be stratified by stage, age and *MYCN* gene status, the most important prognostic factors, and if possible, by chromosome 1p status and ploidy. Non-uniformly distributed therapy also requires stratification.

Goldstein and associates reported increased Pgp more commonly in neuroblastoma after (28%) than before treatment (10%) [12]. They did not correlate *MDR1* expression with outcome. Pgp was determined retrospectively by Northern blot, slot blot or RNase protection analysis (31/49 cases before and 18/49 after therapy, with 11 stage I, 3 stage II, 5 stage III, 1 stage IVS, 9 stage IV, 29 unknown). Positive and negative controls included KB-8-5 (3-fold doxorubicin-resistant, 6-fold vinblastine-resistant HeLa line with *MDR1* RNA signal arbitrarily designated as 30 units), and KB-3-1 (drug-sensitive parent line with undetectable *MDR1* RNA) [71].

Bourhis and associates found increased Pgp more frequently in neuroblastoma after (42%) than before treatment (6%) [11]. In 26 advanced cases studied after treatment, they reported a significantly better response rate to chemotherapy (100 versus 55%; $P = 0.007$) with low-expression (negative or *MDR1* RNA <30 units) than with high-expression (*MDR1* RNA ≥30 units). They did not report on relapse-free and survival rates, median follow-up, or stratify outcome by therapy and prognostic factors. Pgp was determined retrospectively by Northern or slot blot analysis (15/41 cases before and 26/41 after therapy, wth 5 each of stages I, II and III, 4 stage IVS, 22 stage IV). Positive and negative controls included the KB-8-5 and KB-3-1 lines as described by Goldstein and coworkers [71].

We also observed increased Pgp more often in neuroblastoma after (76%) than before treatment (30%), in advanced rather than localised stages, in metastasis rather than primary disease, and in undifferentiated rather than well-differentiated tumours [15, 16, 121]. Only 6% stage III and 63% stage IV, but no early stage or IVS tumours, expressed Pgp initially. In 44 advanced cases studied before treatment (Figure 1), a complete response to chemotherapy (84 versus 46%; $P = 0.0232$) was significantly better with undetectable than any level of increased Pgp, and relapse-free (78 versus 0%; $P < 0.00005$) and survival rates were higher (84 versus 14% at medium follow-up 5.5 years; $P = 0.0002$). With longer median follow-up 6.3 years, Pgp expression remained a significant predictor of outcome in 56 stages III, IVS and IV neuroblastoma.

Pgp was determined retrospectively by multilayer C219 and C494 immunoperoxidase (67 cases before therapy, with 2 stage I, 21 stage II, 17 stage III, 8 stage IVS, 19 stage IV; 21 also after therapy). Positive and negative controls included SKVCR 0.04, 0.1, 0.25, 2.0 ovarian cancer lines (16-, 64-, 510-, 1000-fold multidrug-resistant with Pgp content defined as 2+ to 5+ by staining and by Western blot standardisation), SKVCR 0.015 (very low Pgp control defined as 1+), SKOV3 (drug-sensitive parent cell line with undetectable Pgp), and C219 and C494 isotype-matched controls [72, 122]. We tested consecutive patients with available pretreatment samples, and interpreted results 'blindly'. Uniformly distributed therapy required no stratification. We found that the Pgp effect remained significant despite stratifying individually by stage, age, *MYCN* status, ferritin, Shimada histology and VMA:HVA ratio (P ranging from 0.026 to <0.00005), or simultaneously by stage and age ($P = 0.0011$ and 0.0373, comparing relapse-free and survival rates). Chromosome 1p loss and ploidy were unavailable for stratification.

In contrast to the three previous studies, Nakagawara and coworkers reported similar Pgp expression before (88%) and after treatment (94%), but higher *MDR1* RNA in good-prognosis infant tumours with lower *MYCN* RNA, and lower *MDR1* RNA, in undifferentiated or *MYCN*-amplified stages IV and IVS tumours [66]. In 35 cases of all stages studied before or after treatment, they found a significantly better survival rate (84 versus 14%; $P = 0.0079$) with higher than lower expression (negative or low Pgp levels). However, the significance of this correlation may be questionable since there was no stratification by stage despite there being more advanced tumours in the low-expression group (1 stage I versus 2 stage III, 2 stage IVS and 10 stage IV) than in the high-expression group (2 stage I and 6 stage II versus 8 stage III, 1 stage IVS and 3 stage IV). They did not report response and relapse-free rates, median follow-up, or stratify outcome by therapy and prognostic factors. The investigators determined Pgp retrospectively by slot blot analysis (17/35 cases before and 18/35 after therapy). They included VJ-300 and KB-C1 as positive controls (epidermoid carcinoma lines with *MDR1* RNA signals and resistance levels not stated), and HC-7-5 and C1-R2 as negative controls (drug-sensitive carcinoma line and revertant of KB-C1).

Corrias and coworkers described higher Pgp expression after (33%) than before treatment (17%), and in well-differentiated than undifferentiated tumours, except for stage IVS [123]. In 29 cases of all stages studied before treatment, they found no correlation of Pgp expression with outcome of therapy or stage. They did not report on response, relapse-free and survival rates, median follow-up, or stratify outcome by therapy and prognostic factors. The investigators determined Pgp retrospectively by Northern blot analysis (29 cases before and 3 also after therapy). They did not describe the positive or negative controls used.

Bates and colleagues reported similar *MDR1* RNA (100%) and protein expression (91%) before and after treatment, and higher expression in well-differentiated than undifferentiated tumours [67]. In 11 cases of all stages studied before or after treatment, they found no correlation of Pgp expression with survival, stage, age or site. They did not report on response, relapse-free and survival rates, median follow-up, or stratify outcome by therapy and prognostic factors. The investigators determined Pgp retrospectively by RNase protection, mRNA *in situ* hybridisation and single-layer MRK-16 immunoperoxidase (7/11 cases before and 4/11 after therapy, with 1 stage II, 4 stage III, 6 stage IV). They included the KB-8-5 and KB-3-1 lines as positive and negative controls (*MDR1* RNA signals not stated).

Favrot and associates observed no Pgp expression in neuroblastoma tumour cells before or after treatment, but only in monocytes, histiocytes, fibroblasts, satellite cells, Schwann

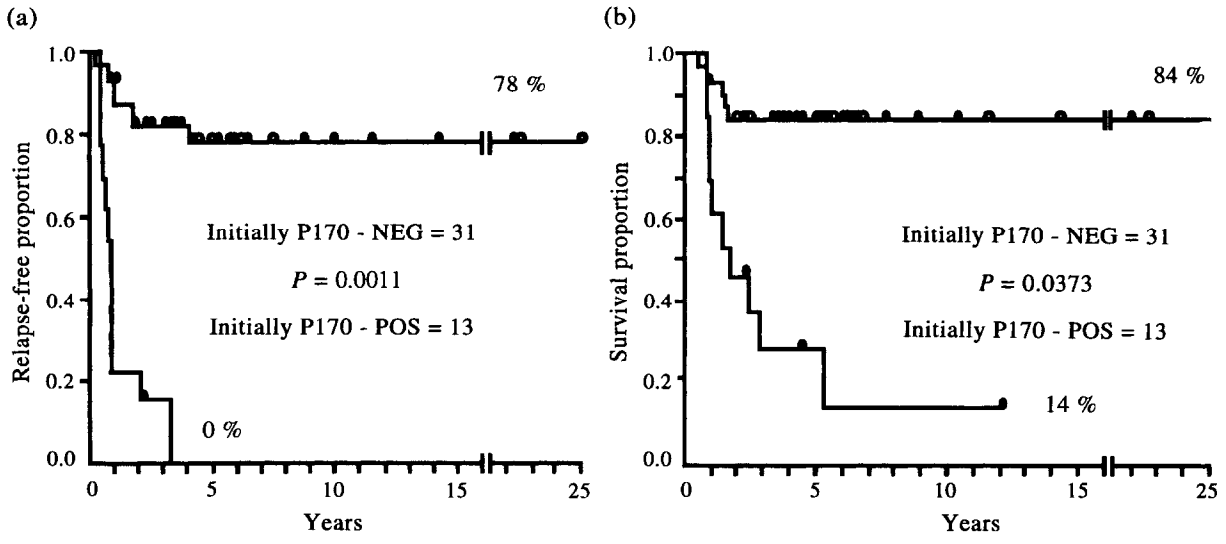

Figure 1. Relapse-free survival (a) and overall survival (b) in 44 patients with non-localised neuroblastoma according to the expression of P-glycoprotein (Pgp) in the tumours at diagnosis. The Kaplan–Meier curves show the probabilities of remaining relapse-free and surviving in 13 patients positive for Pgp (12 stage IV, 1 stage III) and 31 patients negative for Pgp (7 stage IV, 16 stage III, 8 stage IVS tumours). The differences between the relapse-free survival in the two groups ($P < 0.00005$) and overall survival ($P = 0.0002$) were highly significant. These differences remained significant ($P = 0.0011$ and 0.0373, respectively) after log-rank analysis of outcome was stratified simultaneously for age and stage of the tumours. In the group that was negative for Pgp 1 died without relapsing; this event was treated as a censored observation in (a). Reprinted by permission of *The New England Journal of Medicine*, Chan HSL *et al.*, Vol. 325, pp. 1608–1614, 1991. Copyright © 1991 Massachusetts Medical Society. All rights reserved.

cells and adrenal cells. They concluded that increased Pgp does not cause chemotherapy failure [65]. Pgp was determined retrospectively by single-layer C219 immunoalkaline phosphatase (15/37 cases before and 22/37 after therapy, with 5 stage I, 4 stage II, 4 stage III, 5 stage IVS, 19 stage IV). They included the VAC 75 line as positive control (resistance levels and staining versus standardisation of Pgp by Western blot not stated), the C219 isotype-matched control, and peripheral blood lymphocytes as negative control (Pgp-expressing according to most investigators [36, 38]). Since they also included no low Pgp positive control, it is possible that their detection assay was not sensitive enough to detect low levels of Pgp expression.

O'Meara and colleagues also observed no Pgp expression in neuroblastoma tumour cells before or after treatment. They concluded that increased Pgp does not cause chemoresistance [124]. Pgp was determined retrospectively by JSB-1 and MRK-16 avidin-biotin-immunoperoxidase (6/13 cases before and 7/13 after therapy with stages not stated). Positive controls included MCF-7Ad and 2780-Ad (breast and ovarian carcinoma lines with resistance levels and staining versus standardisation of Pgp by Western blot not stated). They did not describe the negative controls used. Since they included no low Pgp positive control, the sensitivity of this detection assay may be questionable.

P-GLYCOPROTEIN EXPRESSION IN RHABDOMYOSARCOMA

Rhabdomyosarcoma and undifferentiated sarcoma are the most common soft-tissue sarcoma in children [125]. Surgery, chemotherapy and radiation are important treatment for these tumours. Localised tumours (groups I–II) have a more favourable prognosis than regional or metastatic tumours (groups III–IV). Embryonal rhabdomyosarcoma do better than the rare alveolar and pleomorphic tumours [126, 127]. Some sites (orbit, non-parameningeal head and neck, pelvis, paratesticular) are more favourable than others (extremity, parameningeal, thorax, torso, retroperitoneum) [126, 127]. We advocate that any correlation of Pgp expression with outcome be stratified by stage and site, the most important prognostic factors, and by therapy if non-uniformly distributed. The prognostic impact is unknown for tumour size, histology, age, sex and lymphocyte count [126].

Chan and associates observed increased Pgp more often in rhabdomyosarcoma after (75%) than before treatment (14%), in advanced rather than localised disease, in metastasis rather than primary tumours, and in unfavourable rather than favourable sites [14, 16, 121]. Only 33% group II, 7% group III, 50% group IV, but no group I tumours were Pgp-positive initially. In 29 cases studied before treatment, response rate to chemotherapy (76% complete and 24% partial versus 50% complete and 50% partial) was better with undetectable than any level of increased Pgp, and relapse-free (76 versus 0% at median follow-up 5.5 years; $P = 0.0009$) and survival rates (80 versus 25%) were higher. Pgp was determined retrospectively as described (29 cases before therapy, with 8 group I, 3 group II, 14 group III, 4 group IV; 12 also after therapy), using the same negative, very low and high positive controls, criteria for interpretation of results, and study methodology [15, 16, 72, 122]. Uniformly distributed therapy and tumour size, histology, age, sex or lymphocyte count required no stratification. We found that the Pgp effect remained significant despite stratifying simultaneously by stage and site ($P = 0.04$, comparing relapse-free rates). The Pgp effect was not significant for response ($P = 0.30$) and survival rates ($P = 0.09$), perhaps because of our small numbers.

O'Meara and associates observed Pgp expressed in 1/5 rhabdomyosarcoma before and 0/1 after treatment. They concluded that increased Pgp was not the cause of chemotherapy failure [124]. The investigators determined Pgp retrospec-

tively as reported previously (5/6 cases before and 1/6 after therapy with stages not stated), using the same positive controls but without negative controls. The absence of a low Pgp positive control makes the sensitivity of this detection assay questionable.

P-GLYCOPROTEIN EXPRESSION IN OSTEOSARCOMA

Osteosarcoma is the commonest bone cancer of children and young adults. Conventional therapy consists of surgery for the primary tumour and adjuvant chemotherapy for systemic micrometastasis [128]. Tumour control is excellent with limb-salvage procedures, amputation or intra-arterial cisplatin therapy. Historically, less than 20% of patients survived without chemotherapy [129]. The most recent multi-institutional trials reconfirmed the importance of chemotherapy [130, 131]. Up to 40% of patients with localised tumours, and >90% with metastatic disease still fail chemotherapy and die [128]. Smaller (<10 cm), well-differentiated (low grade) and distal tumours (distal femur, humerus, tibia, radius, ulna) do better than larger (≥10 cm), undifferentiated (high grade) and proximal tumours (pelvis, proximal femur, humerus) [132]. We advocate that any correlation of Pgp expression with outcome be stratified by tumour size, grade and metastasis, the most important prognostic factors, and by therapy if non-uniformly distributed.

Wunder and colleagues observed a trend toward a poorer outcome in non-metastatic osteosarcoma with higher Pgp expression, at 30-months follow-up [87]. Pgp was determined retrospectively by RT–PCR analysis (15 cases before therapy). Positive and negative controls included KB-8, 8-5, 8-5-11 and 8-5-11-24 which are 1.2-, 6.3-, 51-, 20-fold vinblastine-resistant HeLa lines (with *MDR1* mRNA molecules/cell designated as 2+ to 5+, and as 1+, for signals between KB-8 and 3-1), and KB-3-1 (drug-sensitive parent line with undetectable *MDR1* RNA) [73].

Baldini and colleagues observed increased Pgp in 30% of non-metastatic osteosarcoma before treatment, more often in proximal than distal disease, and in undifferentiated rather than well-differentiated tumours. In 92 cases studied before treatment, the event-free rate (80 versus 40% at a median follow-up of 6 years; $P = 0.002$) was significantly better with low expression (negative or <10% positive cells with weak staining) than high expression (>10% positive cells with weak or strong staining). They did not report on response or survival rates. They found that Pgp status correlated significantly with the event free rate ($P = 0.001$), as did the amount of tumour necrosis (≥90% or <90%) after pre-operative chemotherapy ($P = 0.04$).

The investigators determined Pgp retrospectively by JSB-1 and MRK-16 avidin-biotin-immunoperoxidase. Positive and negative controls included normal kidney and U-2 OS/DX (15-fold multidrug-resistant osteosarcoma line with staining versus standardisation of Pgp by Western blot not stated), and U-2 OS (drug-sensitive parent cell line), but no JSB-1 and MRK-16 isotype-matched controls. They tested consecutive patients and interpreted results 'blindly'. Uniformly distributed therapy required no stratification. However, they did not stratify outcome by tumour size, grade or site (uniformity of distribution not stated).

Chan and associates observed increased Pgp in 44% of osteosarcomas before treatment, more often in metastatic (100%) than non-metastatic (40%) tumours (unpublished data). In patients studied before treatment, favourable responses (≥90% tumour necrosis in 46 given chemotherapy preoperatively) were better (48 versus 17%, $P = 0.057$) with undetectable rather than any level of increased Pgp, and relapse-free and survival rates were significantly higher (87 versus 0% and 94 versus 35% in 61 patients receiving chemotherapy, at median follow-up 8.9 years; both $P < 0.00001$).

Pgp was determined retrospectively as described (61 cases before therapy, with 36 <10 cm and 25 ≥ 10 cm, 3 low grade and 58 high grade, 57 non-metastatic and 4 metastatic), using the same negative/very low/high positive controls, criteria for interpretation of results, and study methodology [14–16, 72, 122]. Uniformly distributed proximal and distal tumour sites required no stratification. We found that the Pgp effect remained significant despite stratifying simultaneously by tumour size, grade and metastasis (both $P < 0.00001$, comparing relapse-free and survival rates). The significant differences in outcome with undetectable rather than increased Pgp were irrespective of whether the patient received 2-drug, 4-drug or >4-drug chemotherapy ($P = 0.00002$, $P = 0.045$ and $P < 0.00001$, comparing relapse-free rates).

OUTCOME OF PAEDIATRIC TRIALS WITH P-GLYCOPROTEIN BLOCKERS

Several experimental approaches attempt to circumvent multidrug resistance due to Pgp. Some investigators have expressed a *MDR1* transgene in bone marrow cells to protect against myelotoxicity from chemotherapy [133]. Other investigators inserted a hammerhead ribozyme in tumour cells to decrease *MDR1* mRNA expression [134]. One group employed low-dose genotoxic mitomycin C to alter promoter function of inducible genes, including *MDR1* [135]. Another group used antibodies (MRK-16, MRK-17, HYB-241) against external Pgp epitopes to prevent efflux of chemotherapy drugs [102, 136, 137]. The most widely tested approach is blocking of the Pgp drug efflux function with verapamil or cyclosporin A. These agents chemosensitise resistant cells by increasing intracellular drug accumulation [138, 139], thereby improving the survival of animals implanted with resistant tumours [140–143].

Several clinical trials have used verapamil and cyclosporin as multidrug resistance blockers for resistant myeloma, lymphoma and acute myeloid leukaemia [10, 13, 54–64]. They all employed prolonged verapamil or cyclosporin infusions (10–26 mg/kg/day) for up to 5 days, and some, also prolonged chemotherapy infusions. To date, although initial response rates in myeloma, lymphoma and acute myeloid leukaemia were better than historical results, cure rates were not improved. High Pgp-expressing colorectal and renal carcinoma showed little response [144, 145]. Even for cancers expressing lower Pgp levels, such as myeloma, lymphoma and leukaemia, chemosensitising verapamil levels are not achievable in blood and tissues because of cardiovascular toxicity [10, 13, 63, 146, 147]. Much higher steady-state blood concentrations (3000–5000 μg/l) occur with prolonged cyclosporin infusions. However, prolonged cyclosporin infusions enhances myeloid, renal, neural and hepatic toxicity and causes hyperbilirubinaemia by altering etoposide or doxorubicin/metabolites clearance, increasing by ≥2-fold their area under the concentration-time curve (AUC) [54–56, 58–61, 148]. Prolonged cyclosporin infusions may also significantly inhibit normal tissue Pgp that is protective at the blood–brain barrier, kidney, liver, and bone marrow haemato-

poietic cells. Prolonged chemotherapy infusions may increase drug AUC and toxicity further.

Of several drug-resistant paediatric tumours we treated with cyclosporin-modulated chemotherapy, the best and most lasting results were in intra-ocular retinoblastoma. Historically, there are no studies showing effective chemotherapy for curing intra-ocular retinoblastoma without irradiation. Only non-visually threatening small tumours away from the optic nerve and macula are curable with focal therapy (laser, cryotherapy, radioactive plaque). Traditionally, medium-sized to large tumours, those with vitreous seeds or ora serrata involvement, or occurring at the optic nerve and macula, require external beam radiation therapy. However, large tumours, vitreous seeds, and ora serrata involvement respond poorly despite radiation. Furthermore, radiation of young children with germline *RB1* mutations incurs a 35% risk of secondary cancers within 30 years, and causes cosmetic deformities and cataracts.

Because we hypothesised that Pgp expressed in retinoblastoma (one-third pretreatment, all that failed) causes chemotherapy failure, we added cyclosporin to vincristine/teniposide ± carboplatin consolidated with focal therapy, to determine whether we could cure intra-ocular tumours without radiation (unpublished data) [52, 149, 150]. We scored patients requiring irradiation, enucleation or macula-destroying focal therapy as failures. In 21 patients, responses were excellent and durable, saving vision and avoiding irradiation or enucleation (overall relapse-free rate 76% at median follow-up 3.3 years; 92% for newly diagnosed tumours). Half of the previously treated, relapsed patients achieved lasting remission with cyclosporin added to the drugs that had failed. Our present results for the worst tumours with vitreous seeds (86% at 3.5 years) were better than published success rates for similar tumours irradiated elsewhere (40% at 6 years) [151], or tumours irradiated and given the same chemotherapy without cyclosporin (45% at 2.6 years). These results are also better than our historical success rate for equivalently poor-risk retinoblastoma treated with similar chemotherapy without cyclosporin, and/or radiation (37% relapse-free rate for 19 patients at median follow-up 5.6 years, $P = 0.032$; 37% for 16 newly diagnosed patients, $P = 0.012$). We saw a better outcome with higher cyclosporin blood levels and projected tissue exposure. Unlike previous trials that gave prolonged cyclosporin infusions and saw greater toxicity, we gave 3 h infusions of even higher cyclosporin doses (33 mg/kg/day) on both days of chemotherapy cycles given every 3 weeks and found low toxicity with few hospital admissions. We saw no increase in myeloid, renal, neural or hepatic toxicity, and no hyperbilirubinaemia with vincristine–teniposide ± carboplatin (comparing frequency of toxicity, average chemotherapy dose intensity, percentage of projected dose intensity ± cyclosporin) [152, 153]. This is the first clinical study to suggest that cyclosporin improves the long-term response to chemotherapy, perhaps by inhibiting Pgp. We are presently proposing a randomised multicentre trial for intraocular retinoblastoma to clarify the role of cyclosporin.

CONCLUSION

The increased expression of Pgp is an important cause of multidrug resistance in tumour cell lines *in vitro*. Whether this mechanism is equally relevant to clinical chemoresistance is still under investigation in many adult and paediatric malignancies. This review has examined the immunohistochemical and molecular biological tools suitable for measuring Pgp in patient samples, interpretation of test results and assessment of study methodology, and reviews the clinical relevance of Pgp in childhood tumours. We discuss the results of a phase I/II retinoblastoma chemotherapy trial modulated by short high-dose cyclosporin infusions. We saw an improved success rate without requiring irradiation of intra-ocular retinoblastoma. Lack of increased toxicity from short high-dose cyclosporin infusions suggests that improved efficacy cannot be entirely due to the inhibition of chemotherapy drug clearance that caused enhanced toxicity in other reversal trials. Poor long-term response in reversal trials of cancers other than retinoblastoma indicates the importance of defining the clinical relevance of redundant multidrug resistance proteins, developing more potent but less toxic reversal agents with broader functional spectrums, and evaluating less toxic modes of reversal agent delivery than those presently used in ongoing trials.

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