In this issue: FLOW CYTOMETRY IN THE CLINICAL LABORATORY

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PREDICTION OF THERAPY RESPONSE AND PROGNOSIS IN LEUKEMIAS BY FLOW CYTOMETRIC MDR ASSAYS

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ABSTRACT

Multidrug resistance (MDR) is an unwanted phenomenon, that may cause therapy failure in several neoplasms including hematological malignancies. The purpose of any type of laboratory MDR assay is to reliably identify such patients and to provide useful data to clinicians with a relatively short turnaround time. MDR can be multicausal and several previous data identified a group of transmembrane proteins - the ATP-binding casette (ABC) proteins - that may be involved in MDR in various hematological malignancies. The prototype of these proteins is the P-glycoprotein (Pgp, MDR1, ABCB1) that is a seven-membrane spanning transmembrane protein capable of extruding several cytotoxic drugs that are of key importance in the treatment of hematological disorders. Similarly other ABC proteins – Multidrug resistance associated protein 1 (ABCC1) and breast cancer resistance protein (ABCG2) are both capable of pumping out cytotoxic drugs. Here, we present flow cytometric methods to identify MDR proteins by antigen and activity assays. The advantage of flow technology is the short turnaround time and its relative easiness compared to nucleic acid based technologies. However, for the activity assays, it should be noted, that these functional tests require live cells, thus adequate results can only be provided if the specimen transport can be completed within 6 hours of sample collection. Identification of MDR proteins provides prognostic information and may modulate therapy, thus signifies a clinically useful information in the evaluation of patients with leukemias.

INTRODUCTION

Drug resistance may be intrinsic or acquired and it severely impairs the progress in cancer chemotherapy [1,2]. The importance of this phenomenon is underlined by the fact that its presence is not limited to malignancies but may hamper the success of therapy in several other diseases like rheumatoid arthritis and epilepsy. In MDR, resistance occurs to several chemically unrelated drugs, lipid-soluble drugs like anthracyclines, vinca alkaloids, epipodophyllotoxins, antibiotics and the resistance can be caused by one or more of several mechanisms. A frequently observed phenomenon is when the drug is quickly extruded from the cell by transporters before any cytotoxic action can be elicited. These efflux proteins are localized in the cell membrane, however further intracellular sites were also described and these are thought to contribute to resistance by accumulating the drug in intracellular compartments and preventing it from reaching its nuclear targets [3].
The best studied efflux pump is a permeability glycoprotein (P-glycoprotein, Pgp) which is a 170 kDa protein that cleaves ATP to cover the energy needed for expelling many xenobiotics. Pgp consists of two homologous halves, each consisting of six transmembrane segments and one ATP-binding domain. The most accepted model is based on the presence of a catalytic

intermediate where both catalytic sites of Pgp are active and ATP is hydrolysed alternatively alternatively (Figure 1.). ATP hydrolysis at one site triggers conformational changes within the protein resulting in drug transport, while hydrolysis of a second ATP at the other site is required for resetting the original high- affinity binding conformation [4-7]. The transmembrane segments containing the drug-binding site are quite mobile so drug binding occurs through a substrate-induced fit mechanism. This mechanism explains how Pgp can accommodate a broad range of compounds [7]. Pgp was designated as MDR1 and it was shown 25 years ago, that the expression of a full-length cDNA for the human "MDR1" gene confers resistance to colchicine, doxorubicin, and vinblastine [8]. The human Pgp belongs to a large group of transport proteins, known as ATP-binding casette (ABC) superfamily, that share common structural and functional properties. To date, about 50 human ABC transporter genes have been identified. Their protein products are classified into seven groups entitled: ABCA-ABCG.

In addition to MDR proteins other membrane pumps extrude somewhat different substrates than Pgp. A 190 kDa protein called MRP (multidrug resistance related protein) is a group of transporters that can contribute to clinical resistance. In contrast to Pgp, MRP1 expression is predominant in the basolateral plasma membrane. MRP protein functions as a multispecific organic anion transporter, with oxidized glutathione, cysteinyl leukotrienes, and activated aflatoxin B1 as substrates. This protein also transports glucuronides, sulphate conjugates of steroid hormones, bile salts and other hydrophobic compounds in the presence of glutathione [9-12]. MRP proteins play an important physiological role in the protection of the body against xenobiotics occurring in the environment. MRP2 and MRP3 seem to play a role in organic conjugate transport while MRP4 and MRP5 may have a nucleotide transporter function.

The third most studied efflux protein is the breast cancer resistance protein (BCRP, ABCG2). Its mRNA encodes this 663 amino acid member of the ATP-binding cassette superfamily of transporters. Enforced expression of the full-length BCRP cDNA in MCF-7 breast cancer cells confers resistance to mitoxantrone, doxorubicin, and daunorubicin, reduces daunorubicin accumulation and retention, and causes an ATP-dependent enhancement of the efflux of rhodamine 123 in the cloned transfected cells [13]. Furthermore its over-expression was identified as a negative prognostic marker in acute myeloid leukemia patients and it was described that the survival significantly worsened in case of BCRP over-expression concomitant with Pgp and other unfavourable prognostic markers. [14, 15].

Importance of drug resistance in hematological malignancies

The importance of MDR in hematological malignancies seem to be well-established, since from the historic paper of Ueda et al. in 1987 over 1400 hits can be found in Medline about MDR and leukemias. The mechanisms that may contribute to the enhanced Pgp expression are the activity of its transcription factor [16], gene rearrangement [17], or hypomethylation of the mdr-1 promoter region [18]. In addition, Pgp has been investigated in lymphoid malignancies and it has been demonstrated that patients with increased level of Pgp, either at diagnosis or upon relapse have poorer prognosis than those patients who do not express Pgp [19-21]. Undoubtedly however, the majority of literature deals with MDR in AML patients, where drug resistance is primarily determined by Pgp [22-24] although other transporters were also found to have significance [25-27].

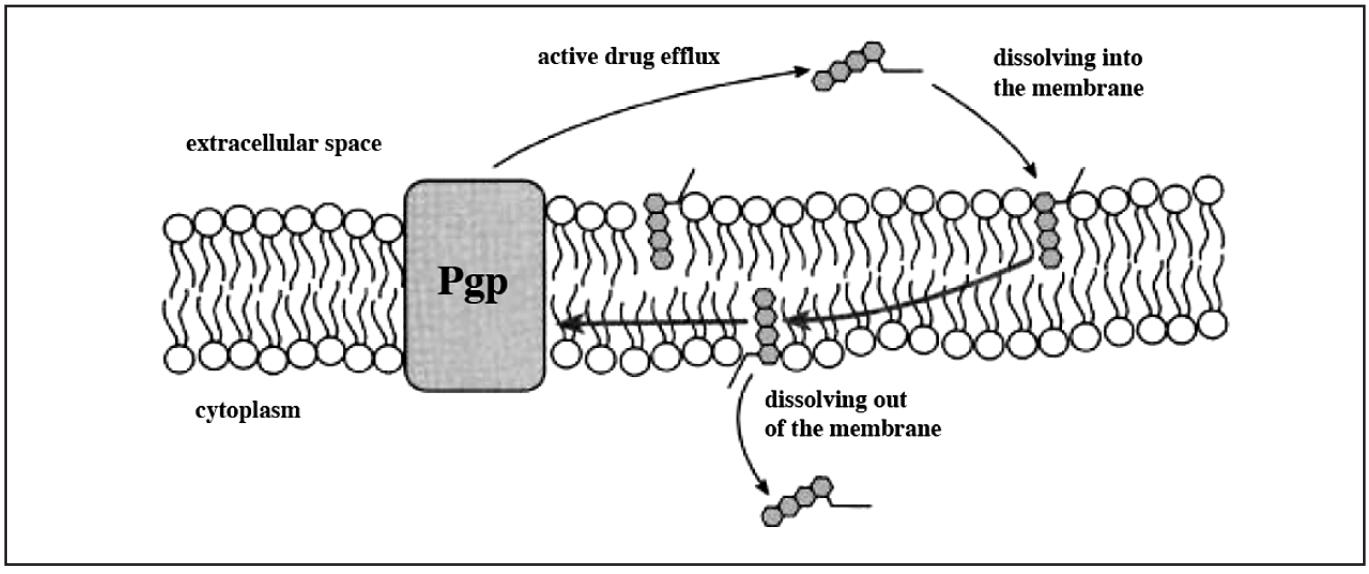

Figure 1
Function of MDR proteins. Drug-efflux proteins like Pgp expel xenobiotics already from the cell membrane, thus most of these molecules can not enter the cell.

Aside from de novo resistance, MDR can develop during chemotherapy as sensitive cells are killed off and genetic resistance is induced either by the chemotherapy itself or emerges spontaneously during treatment. Although normal tissues possess constitutive Pgp activity, acquired resistance never develops in noncancerous cells. Pgp may not only influence disease outcome as a pump protein, but because of its involvement as an intramembranal substrate transport, it may have anti-apoptotic effect as well.
In addition to the analysis of MDR pump function at the protein level, several studies have lead the way to delineate the role of MDR-1 genetic polymorphisms in disease outcome. MDR-1 single nucleotide polymorphisms were found to be associated with an achievement of complete remission and event-free survival in AML patients, however they were not found to be associated with overall survival [28].

Methods to detect drug resistance

A few considerations are important when MDR diagnostic assays are discussed. One is that in hematological malignancies more than one mechanism of drug resistance may be present that may impose a special diagnostic problem. The other is that most of the data on MDR assays are derived from measurements on cell lines. However in clinical samples the efflux proteins like Pgp is present in several orders of magnitude lower copy numbers. There are three different approaches to identify MDR.

(i) RNA measurement for different MDR proteins
(ii) determination of protein expression by flow cytometry or immunohistochemistry
(iii) functional tests that measure the transport activity of MDR proteins.

The 3-4,5 dimethylthiazol-2-yl-2,5-diphenyltetrazolium-bromide (MTT) assay or that is more simply referred to as cell survival assays is used in some laboratories. It reports results in quantitative terms, however it is laborious and requires 4 days and thus is unsuitable for routine analysis of clinical samples. According to a consensus paper published in 1996 [29] two methods have to be executed in order to obtain reliable results for MDR testing. The problem with RNA measurement is that mRNA level does not necessarily correlate with the expression of the relevant proteins and the mRNA results are often provided only in semi-quantitative terms.

MDR antigen and activity measurements

Antigenic assays are easy to execute and thus can easily fit into the general routine of a flow cytometry laboratory as all CD markers are detected by using directly conjugated antibodies. However, the major drawback of antigenic detection of MDR is that the different clones of antibodies have variable sensitivity and thus the results obtained are often highly variable. Furthermore, reporting of MDR antigenic data is not straightforward as in many cases the conventional percent positivity is not very meaningful due to the low expression rate in clinical samples. Thus, authors report the results in terms of fluorescence units or mean fluorescence intensities. Here, the ratio of the sample mean fluorescence channel and the isotypic control fluorescence channel was calculated and MFI ratios exceeding values found on normal blood cells were reported as positive. Thus, relative fluorescence intensity values (RFI) are usually provided for various MDR proteins when measured by antibodies [30]. Some papers, however points to the possibility that certain antibodies detecting MDR1 have been shown to be sensitive to conformational changes [31,32], thus an increase in antibody binding capacity may be exploited in the investigation of clinical samples. Functional assays definitely offer an advantage over antigen measurements since they measure the clinically relevant property, the transport activity of MDR proteins. The most widely used functional assay to detect MDR activity is based on the measurement of a fluorescent substrate. These are mainly accumulation type assays where the fluorescent dye is continuously accumulated in the cell either due to its binding to intracellular structures like doxorubicin or due to its intracellular enzymatic modification like in case of calcein-acetoxymethyl ester. The fluorescence tracers in the majority of these assays are the rhodamine 123 [33], calcein-AM, [34] or JC1 [35]. The tracer JC1 has proven to be useful for the simultaneous detection of Pgp activity and apoptosis in leukemic cells [36]. These functional assays are evaluated by flow cytometry and the principle of the measurement is to measure fluorescence of cells in the presence and absence of efflux pump inhibitors. An advantage of functional assays is, that they can be combined with surface staining of the leukemic cells, thus the cell population of interest can be gated out [37] by labelling the sample by the appropriate antibody and the efflux activity of the desired cells population is analysed with and without the efflux pump inhibitor (Figure 2). It should be noted that in this assay the positive population is represented with the lower fluorescence values– since the indicator dye is removed from the cells – while in an antigen assay the positive cells are always displayed with higher fluorescence values as observed with any other flow cytometric antigen assay (Figure 3.).
The quantitative results of the functional assay can be expressed in multidrug resistance activity factor (MAF) units by using selective inhibitors. The difference in fluorescence is proportional to the activity of the efflux pump. In a typical flow activity assay, MDR1 and MRP1 are inhibited separately and thus total MDR activity can more appropriately be dissected. A definite

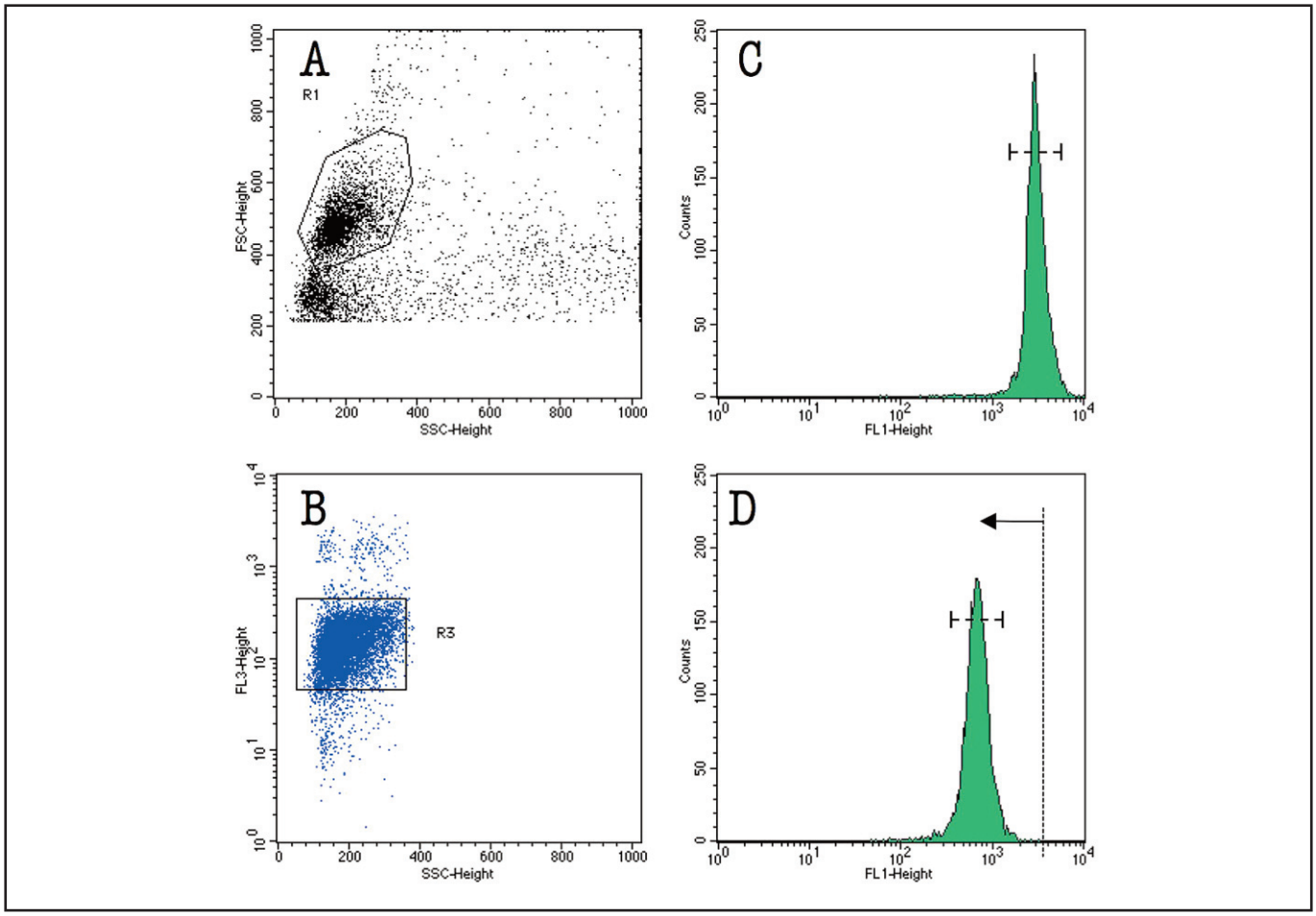

Figure 2
Functional MDR assay in an AML sample. The cells are identified on the FS-SS dot plots (panel A) than subsequently the CD45-dim myeloid blasts are gated out (panel B). Calcein is measured on the blasts in the presence (panel C) or absence (Panel D) of an MDR inhibitor. Note that the fluorescence values are lower in the absence of the inhibitor.

disadvantage of the functional assays, however that they require living cells, thus prolonged storage of samples is not possible. This is probably one reason why the detection of MDR in flow cytometry laboratories is relatively rare.
In our previous work the functional assay was correlated to an antigenic assay carried out by quantitative flow cytometry [38]. We performed the determination of the antibody binding capacity by incubating cells with the anti-Pgp antibody MRK16 and subsequently with a FITC labeled anti-mouse IgG. Results were expressed as ABC by using a calibration curve obtained by measuring the fluorescence intensity of precalibrated beads. The multidrug resistance activity factor (MAF) values were determined with the MDR inhibitor Verapamil (Vp). In triplicate samples by the preincubation of samples with (Vp+) or without (Vp-) the MDR blocker and subsequently loading them with calcein-AM. The calcein fluorescence was measured and the values MFI (Vp+) – MFI (Vp-)/MFI (Vp+) x100 were reported as the MAF value corresponding to MDR activity. This quantitative measurement of MDR activity is characteristic for the cumulative activity of Pgp and MRP1 as verapamil inhibits both transporters. When MAF was determined in the presence of the MRP1 inhibitor MK571 the MAF_{Vp}-MAF_{MK571} value referred to the Pgp specific resistance.
We found that in the high Pgp expressor KB-V1 cell line an extremely high MDR activity was detectable along with high number of Pgp molecules/cell while in the low expressor KB-8-5 cell line the functional assay resulted only in a 20% decrease while the number of Pgp molecules decreased by over 90%. This also refers to the better sensitivity of the functional assay.

A commercially available kit is available that measures the activity of multiple transporters. Preliminary results with this MultiDrugQuant assay kit were published and the authors were able to show a significant correlation between the expression of the multidrug resistant proteins (P-gp and MRP1) and their functional activity in adult AML and pediatric ALL samples [39]. This test was also suitable to identify drug resistance in solid tumors as collagenase disintegration preserved the MDR activity and the antigenicity of tumor cells. The extended calcein assay provided sufficient viable and functionally active tumor cells

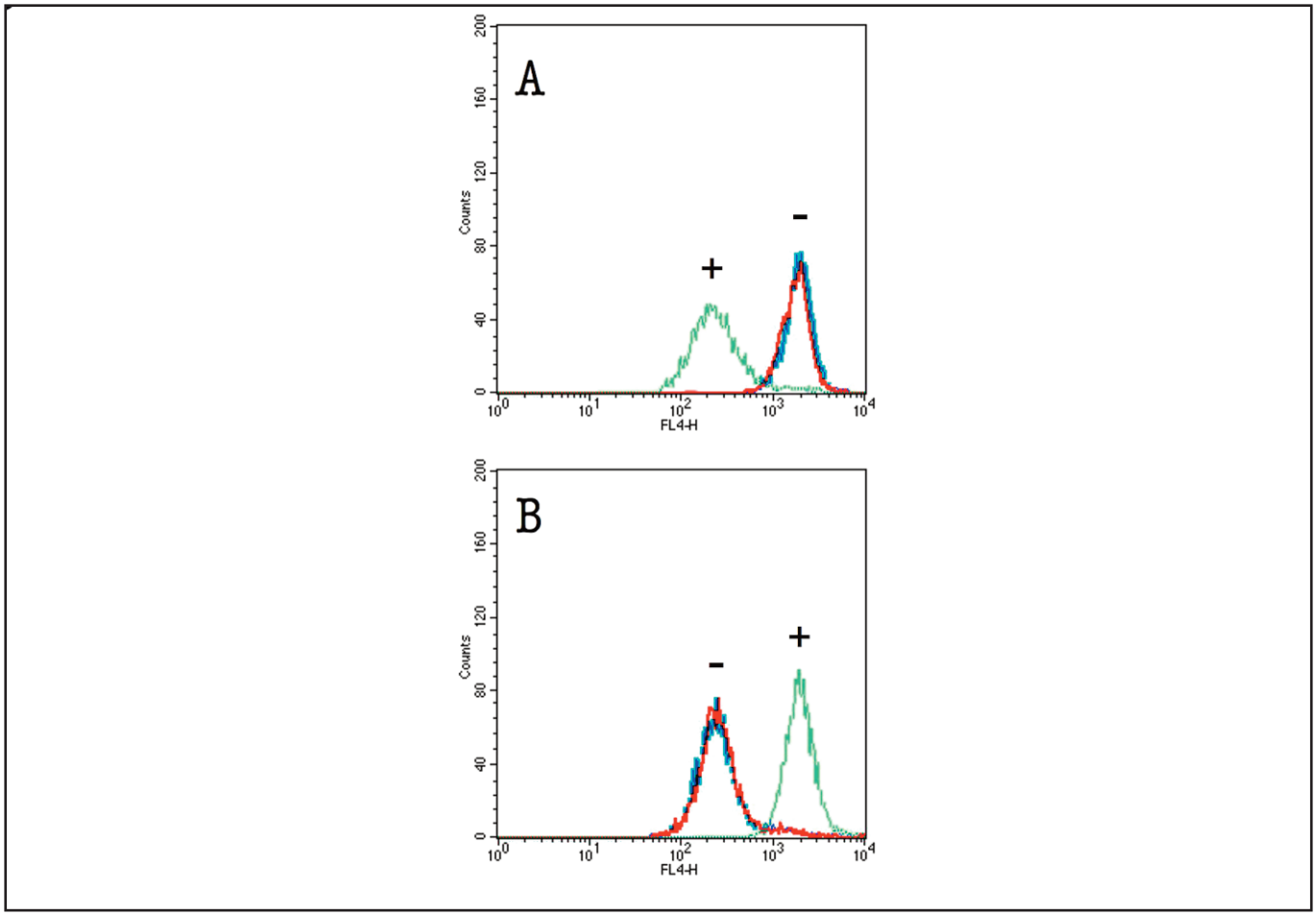

Figure 3
Comparison of the functional an antigen assays in cell lines. In the functional MDR assays (panel A) positive cells display reduced fluorescence values since they expel the fluorescent dye. In the antigen assays (panel B) the positive cell line displays higher fluorescence due to binding the fluorescent antibody.

from surgical biopsies to determine the functional MDR activity [40].
In 2011 the SOLVO-MDQ kit received a CE/IVD qualification. It provides all necessary ingredients to execute functional MDR assays for Pgp, MRP1 and BCRP by using selective inhibitors and two different fluorescence dyes and as a minimum requirement a 4-color flow cytometer is required.

Conclusions

Flow cytometry is increasingly been used in clinical laboratories and today already small benchtop cytometers can provide the advantage of using multiple colors. We anticipate that in hematology, as many novel probes emerge functional assays may become more popular and one such application is likely to be the detection of MDR protein activities, that is easier with a CE-marked diagnostic kit.

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