HET PROGNOSTISCH BELANG VAN P-GLYCOPROTEINE BIJ KINDEREN MET ACUTE LYMFOBLASTEN LEUKEMIE EN NEUROBLASTOMEN

Faculteit Geneeskunde en Gezondheidswetenschappen
Dienst Pediatrie

HET PROGNOSTISCH BELANG VAN P-GLYCOPROTEINE BIJ KINDEREN MET ACUTE LYMFOBLASTEN LEUKEMIE EN NEUROBLASTOMEN

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Proefschrift voorgedragen tot het behalen van de graad van
Doctor in de Medische Wetenschappen

Promotors: Prof. Dr. G. Laureys en Prof. Dr. J. Philippé

2002

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Het onderzoek, beschreven in dit proefschrift, werd uitgevoerd in de laboratoria van de afdeling Pediatrische Hemato-Oncologie, van de dienst Klinische Biologie, Microbiologie en Immunologie en van de dienst Medische Genetica, en eveneens in de dienst Nucleaire Geneeskunde van het Universitair Ziekenhuis Gent.

Deze studie kwam tot stand dankzij een mandaat Aspirant van het Fonds voor Wetenschappelijk Onderzoek (F.W.O.) Vlaanderen aan Barbara De Moerloose en Onderzoeksproject nr. 3.0105.95 van het F.W.O. Vlaanderen.

Voor mijn ouders
Voor Johan en onze kinderen,
Charlotte, Frederik, Andreas en Alexander

DANKWOORD

Het tot stand komen van dit doctoraat zou niet mogelijk geweest zijn zonder de steun en de hulp van velen. In 1994 vroegen Prof. Dr. Leroy en Dr. Katrien Dhooge mij om mee te werken aan het onderzoek naar P-glycoproteïne. Beiden hebben mij jarenlang begeleid en het project in goede banen geleid. Dit was eveneens de verdienste van Prof. Dr. Yves Benoit, Prof. Dr. Geneviève Laureys en Prof. Dr. Jan Philippé, waarvoor dank. Ook wil ik de andere leden van de begeleidingscommissie en de leescommissie danken voor hun doordachte opmerkingen en nuttige tips om het doctoraatsproefschrift te kunnen afwerken.

Een belangrijk deel van mijn tijd bracht ik door in het laboratorium van de Pediatrische Hemato-Oncologie. Een speciaal woord van dank wil ik dan ook uitspreken voor Jeannine Geerinck. Zij hielp mij vaak met het praktische werk en was steeds een van mijn vurigste supporters.
Prof. Dr. Kint wil ik danken voor zijn hulp bij mijn eerste kennismaking met laboratoriumwerk. Zijn kritische geest hielp om een degelijke basis te leggen voor het onderzoek. Ook Lucrèce De Buck, Marleen Vandenberghe en Edith George van het labo van de Pediatrie zorgden voor allerlei praktische hulp en van tijd tot tijd voor de nodige morele steun.

Een echte doorbraak in het onderzoek kwam er door de samenwerking met Prof. Dr. Jan Philippé van de dienst Klinische Biologie en Dr. Christophe Van de Wiele van de dienst Nucleaire Geneeskunde. Hun enthousiasme en nieuwe ideeën hielpen om het doctoraat te kunnen voltooien. Uiteraard wil ik ook alle laboranten van het laboratorium Hemato-Immunologie bedanken, in het bijzonder Odette Claeys, Chris Francart, Nadine Van Landschoot, Chris De Ridder en Lutgarde De Wulf, evenals Prof. Dr. Dierckx en Jean-Pierre Van Haelst van de dienst Nucleaire Geneeskunde.
Mijn oprechte dank gaat ook uit naar Prof. Dr. Speleman, Dr. Nadine Van Roy en alle "weefselkwekers" van de dienst Medische Genetica.

Doorheen de jaren heb ik vele assistenten leren kennen, zowel op de dienst Klinische Biologie als Pediatrie. Een tijdlang leek het zelfs of ik eerder klinisch bioloog in plaats van pediater in opleiding was. Ik wil hen hartelijk danken voor de hulp, voor hun interesse en bemoedigende woorden en de talloze ontspannende momenten.
In het bijzonder wil ik ook Katrien Swerts bedanken, die reeds een drietal jaar vol overgave meewerkt aan het MDR-project. Haar enthousiasme en ijver zullen ook in de toekomst een belangrijke stimulans zijn.

Tenslotte wil ik mijn hele familie danken. Mijn ouders leerden mij hoe je via werklust, volharding en doorzettingsvermogen dromen kan waarmaken. Zij steunen en motiveren mij en aarzelen nog steeds niet om in de bres te springen. Mijn man, Johan, verdient ook een bijzonder woord van dank. Ons leven is nooit alledaags geweest en tegenwoordig eerder hectisch. Ongetwijfeld komen er in de toekomst nieuwe uitdagingen in ons leven, maar toch denk ik dat we bij het beëindigen van dit "doctoraat-hoofdstuk" terug iets meer tijd hebben om te genieten van ons prachtig gezin.

Gent, september 2002

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AFKORTINGEN

ABC	ATP-bindingscassette
ABR	abnormally banding region
ALL	acute lymfoblasten leukemie
AML	acute myeloïde leukemie
ANLL	acute niet-lymfoblasten leukemie
APAAP	alkalisch fosfatase-anti-alkalisch fosfatase
ARA	anthracycline resistance-associated protein
ATP	adenosine trifosfaat
BCRP	breast cancer resistance protein, P-95, MXR, ABCP, ABCG2
BDNF	brain-derived neurotrophic factor
BENG	Belgian Neuroblastoma Group
BSPHO	Belgian Society for Paediatric Haematology and Oncology
cDNA	complementair DNA
CFTR	cystic fibrosis transmembrane regulator
CML	chronische myeloïde leukemie
c-MOAT	canalicular multispecific anion transporter
CR	complete remissie
CT scan	computer tomografie scan
Dms	double minutes
DNA	deoxyribonucleïnezuur
EFS	event-free survival
EORTC-CLCG	European Organisation for Research and Treatment of Cancer-Childhood Leukemia Cooperative Group
FISH	fluorescentie in situ hybridisatie
GST	glutathion-S-transferase
HSR	homogeneously staining region
INES	Infant Neuroblastoma Study
INSS	International Neuroblastoma Staging System
LDH	lactaatdehydrogenase
LNESG	Low Stage Neuroblastoma Study Group
LOH	loss of heterozygosity
LRP	lung resistance protein, lung cancer related protein
MDR	multidrugresistentie
MDR1	multidrugresistentie 1 gen
MDR3	multidrugresistentie 3 gen
MIBG	metaiodobenzylguanidine
MoAb	monoklonaal antilichaam
MRD	minimale ziekterest
MRI	magnetic resonance imaging
mRNA	messenger RNA
MRP	multidrugresistentie-geassocieerd proteïne
MYCN	N-myc oncogen
NGF	nerve growth factor
NSE	neuron-specifiek-enolase
NT	neurotrofine
OS	overall survival

P-gp	P-glycoproteïne, het MDR1 genproduct
Rho 123	Rhodamine 123
RNA	ribonucleïnezuur
RT-PCR	reverse transcriptase polymerase chain reaction
SIOP	Société Internationale d’Oncologie Pédiatrique (International Society of Paediatric Oncology)
sP-gp	sister of P-glycoprotein
TAP	transporter associated with antigen-presentation
^{99m}Tc sestamibi	Technetium-99m hexakis 2-methoxyisobutylisonitrile (sestamibi)
TRK	tyrosine kinase
VIP	vasoactief intestinaal peptide
WHO	Wereldgezondheidsorganisatie

SAMENVATTING

P-glycoproteïne (P-gp), een eiwit met een "pompfunctie" gelegen in de celmembraan van sommige maligne cellen, is verantwoordelijk voor het verwijderen van bepaalde cytostatica uit de cel waardoor die cel hieraan resistent wordt. Dit fenomeen wordt multidrugresistentie (MDR) genoemd. P-gp kan gebruikt worden als aangrijpingspunt van bepaalde inhibitoren waardoor MDR omzeild wordt.

Dit proefschrift is een zoektocht naar het prognostisch belang van het MDR eiwit P-gp bij kinderen met een acute lymfoblasten leukemie (ALL) of een neuroblastoom.

Het eerste hoofdstuk is een literatuurstudie waarin beschreven wordt hoe resistentie aan chemotherapie kan ontstaan. De structuur van P-gp, het voorkomen in normale weefsels en in tumoren, het werkingsmechanisme en de functie ervan worden toegelicht. Ook op de werking van P-gp inhibitoren, de regulatie van zijn expressie en enkele andere vormen van cellulaire resistentie, zoals het multidrugresistentie-geassocieerd proteïne (MRP), wordt ingegaan.
De bekende prognostische parameters van ALL en van het neuroblastoom worden beschreven en voor beide aandoeningen wordt een literatuuroverzicht van het belang van P-gp gegeven.

In hoofdstuk 2 worden de in vitro en in vivo detectietechnieken van P-gp, die gebruikt worden in dit proefschrift, toegelicht: de immuuncytochemische APAAP techniek, de immunologische en functionele flowcytometrische testen en een scintigrafische functionele test met behulp van het isotoop Technetium-99m sestamibi (^{99m}Tc sestamibi).
Er wordt een grondige vergelijking van de immuuncytochemische en flowcytometrische testen gemaakt bij ALL op kinderleeftijd. De flowcytometrische functionele test wordt, op basis van internationale aanbevelingen, als gouden standaard beschouwd. De gevoeligheid en specificiteit van de immuuncytochemische en flowcytometrische immunologische test worden nagegaan. Op basis van deze vergelijking van technieken wordt de combinatie van de flowcytometrische functionele test met de immuuncytochemische APAAP techniek als meest informatieve, en dus meest relevante, beschouwd.
Ook de toepassing van de flowcytometrische testen op twee patiënten met chronische myeloïde leukemie (CML) wordt in dit hoofdstuk beschreven. Deze patiënten worden op verschillende tijdstippen tijdens chemotherapie of bij recidief getest en er worden pas P-gp positieve blasten teruggevonden naarmate er meer chemotherapie toegediend werd. Derhalve is het beter om patiënten die in aanmerking zouden kunnen komen voor een behandeling met een P-gp inhibitor, niet enkel bij diagnose te testen, maar ook tijdens de therapie.

De behandeling van ALL patiënten wordt aangepast aan de hand van verschillende prognostische parameters, zoals aantal witte bloedcellen bij diagnose, reactie op behandeling met prednisolone, leeftijd van de patiënt, immuunfenotype van de lymfoblasten, chromosomale afwijkingen en minimale ziekterest (MRD). Toch zullen nog ongeveer 25 % van de kinderen recidiveren. In hoofdstuk 3 worden stalen van kinderen met ALL nagekeken door middel van de immuuncytochemische techniek enerzijds en de combinatie van deze test met de functionele flowcytometrische test anderzijds. Via prospectieve studies wordt nagegaan of P-gp een prognostische parameter is bij ALL op

kinderleeftijd en welke test (of testcombinatie) ter detectie van P-gp de beste voorspelling van recidief kan geven.
In een eerste studie wordt P-gp immuuncytochemisch opgespoord met de monoklonale antilichamen (MoAbs) C219 en 4E3, en blijkt dat de EFS van de P-gp positieve kinderen significant lager is dan deze van de patiënten negatief voor P-gp. In een tweede studie blijkt P-gp prognostisch relevant te zijn wanneer de immuuncytochemische techniek (MoAbs 4E3 en MRK16) gecombineerd wordt met de functionele flowcytometrische test.

In hoofdstuk 4 worden de verschillende in vitro en in vivo P-gp detectietechnieken op stalen van kinderen met een neuroblastoom, ganglioneuroblastoom of een ganglioneuroom toegepast. Eerst wordt immuuncytochemisch onderzocht bij welke patiënten P-gp expressie voorkomt, of dit correleert met andere prognostische parameters en bovendien hoe de overleving is van de P-gp positieve patiënten. In deze studie kan géén duidelijk prognostisch belang van P-gp expressie bij neuroblastomen teruggevonden worden.
Nadien wordt een flowcytometrische multiparametrische test toegepast op neuroblastoomcellijnen, beenmergstalen geïnvadeerd door neuroblasten en gesuspendeerde tumorcellen. P-gp expressie wordt in deze stalen ook immuuncytochemisch nagegaan en bovendien wordt P-gp functie flowcytometrisch onderzocht. In deze studie wordt vooral een discrepantie tussen de sterkte van P-gp expressie en P-gp pompfunctie vastgesteld. P-gp wordt zeer frequent teruggevonden in de lage neuroblastoomstadia en de beter gedifferentieerde tumoren. Ook na histologische uitrijping door chemotherapie worden P-gp expressie en activiteit aangetoond. Er wordt géén verband gezien tussen de P-gp status van de tumor en de uiteindelijke prognose van de neuroblastoompatiënten.
De toepassing van het in vivo beeldvormingsonderzoek met ^{99m}Tc sestamibi, dat een substraat voor P-gp is, bij neuroblastoom- en ganglioneuroompatiënten en de vergelijking van dit onderzoek met de flowcytometrische testen wordt eveneens in dit hoofdstuk beschreven. De afwezigheid van ^{99m}Tc sestamibi retentie in het merendeel van de tumoren komt overeen met de in vitro aanwezigheid van een actieve P-gp pomp. Een praktisch gevolg hiervan is dat beeldvorming met ^{99m}Tc sestamibi niet bruikbaar is om neuroblastaire tumoren te detecteren. Anderzijds kunnen ^{99m}Tc sestamibi scans niet aangewend worden om klinische MDR te voorspellen.

In tegenstelling tot de kinderen met ALL waar wél een significant verband gevonden wordt tussen P-gp aanwezigheid en een toegenomen kans op recidief, is dit bij neuroblastomen niet het geval. De resultaten van het onderzoek suggereren eerder een verband tussen P-gp expressie en maturatie of differentiatie van deze tumorcellen.

SUMMARY

P-glycoprotein (P-gp), a pump located in the plasma cell membrane, extrudes several clinically important drugs from the cell, and hence causes multidrug resistance (MDR). Reversing clinical drug resistance is possible by using agents that inhibit the activity of P-gp. The main goal of this thesis is the elucidation of the prognostic relevance of the MDR protein P-gp, in childhood acute lymphoblastic leukaemia (ALL) and neuroblastoma.

The emergence of chemotherapy resistance, the structure and function of P-gp and the regulation of its expression, and the current knowledge on the multidrug resistance-associated protein (MRP) are described in the first chapter. The role of P-gp as a potential prognostic factor in childhood ALL is discussed. Since a substantial number of stage 4 neuroblastoma patients acquire drug resistant disease during chemotherapy, a review of the literature about the prognostic parameters in neuroblastoma and about the possible prognostic value of P-gp in this tumour is made.

Chapter 2 is dedicated to the P-gp detection assays which are applied in this thesis: the immunocytochemical APAAP assay, the immunological and functional flow cytometric assays and an imaging technique using the P-gp substrate Technetium-99m sestamibi (^{99m}Tc sestamibi).
A comparison of all in vitro detection assays is made in childhood ALL. The combination of immunocytochemistry with the flow cytometric functional assay is being promoted as the most sensitive and clinically relevant amongst the different techniques.
Two patients, suffering from chronic myeloid leukaemia (CML), are tested sequentially during chemotherapy and become P-gp positive during treatment. This finding stresses the importance of sequential analyses of P-gp, in particular if the patients might benefit from a treatment with a P-gp inhibitor.
Chapter 3 focuses on the prognostic relevance of P-glycoprotein in childhood ALL. In the first prospective study, the immunocytochemical APAAP assay with the monoclonal antibodies (MoAbs) C219 and 4E3 is being applied. Children scoring positive for either one of the MoAbs or for both MoAbs have a significantly worse outcome than those with a negative test result. In a second prospective study, the prognostic relevance of P-gp in childhood ALL is being confirmed, using the combination of immunocytochemistry (MoAbs 4E3 and MRK16) and the functional flow cytometric assay.

In chapter 4, P-gp is examined in neuroblastoma, ganglioneuroblastoma and ganglioneuroma by different detection assays: immunocytochemistry, flow cytometric immunological and functional tests and an in vivo imaging technique using ^{99m}Tc sestamibi.
Immunocytochemistry alone does not provide a prognostic role for P-gp in neuroblastoma. On the contrary, using flow cytometric tests, many neuroblastomas score positive and a discordance is found between the expression and activity level of P-gp. P-gp is found more frequently in low-stage neuroblastoma, differentiated tumours and tumours after chemotherapy. A good correlation between flow cytometric results and imaging results is seen. Consequently, ^{99m}Tc sestamibi scintigraphy is not useful as an in vivo predictor of MDR in neuroblastoma. Unlike the findings in childhood ALL, P-gp does not contribute to MDR in neuroblastoma but seems to be a marker of differentiation.

RESUME

La P-glycoproteïne (P-gp) agit comme une pompe membranaire capable d'expulser des cytostatiques hors de la cellule, et de causer ainsi une résistance aux médicaments anticancéreux. La détection de P-gp est importante en clinique, car on peut administrer des modulateurs qui bloquent cette pompe en même temps que les cytostatiques. Le but principal de cette thèse est de déterminer l'importance pronostique de P-gp dans la leucémie lymphoblastique aiguë (LLA) de l'enfant et dans le neuroblastome.

Le phénomène de résistance à la chimiothérapie, la découverte de P-gp, sa structure, sa fonction et son rôle, ainsi que quelques autres modes de résistance cellulaire sont décrits dans le chapitre 1. L'importance clinique de la présence de P-gp dans la LLA de l'enfant et le neuroblastome, déjà publiée, est discutée, ainsi que les autres paramètres pronostics.

Plusieurs techniques qui permettent d'étudier non seulement l'existence de l'expression structurelle de P-gp mais aussi sa fonction sont décrits au chapitre 2: la technique immunocytochimique (APAAP), l'étude immunologique et fonctionelle par cytométrie de flux et la scintigraphie au Technetium-99m sestamibi (^{99m}Tc sestamibi), un substrat de la P-gp.
Les corrélations entre la technique immunocytochimique et les études cytométriques sont abordées. En particulier, la combinaison de la méthode APAAP avec l'examen cytométrique fonctionnel est recommandée.
Les résultats successifs de l'expression et de l'activité de P-gp en cytométrie de flux chez deux patients chez lesquels on assiste à la transformation lymphoblastique aiguë d'une leucémie myéloïde chronique sont présentés. Au début de la phase aiguë l'expression de P-gp n'était pas détectable. Par contre, après un traitement par agents anticancéreux, une expression significative était démontrée. Pour la sélection précise des patients qui pourraient bénéficier d'un traitement avec modulateurs, des études répetées de P-gp au cours de la leucémie sont indispensables.

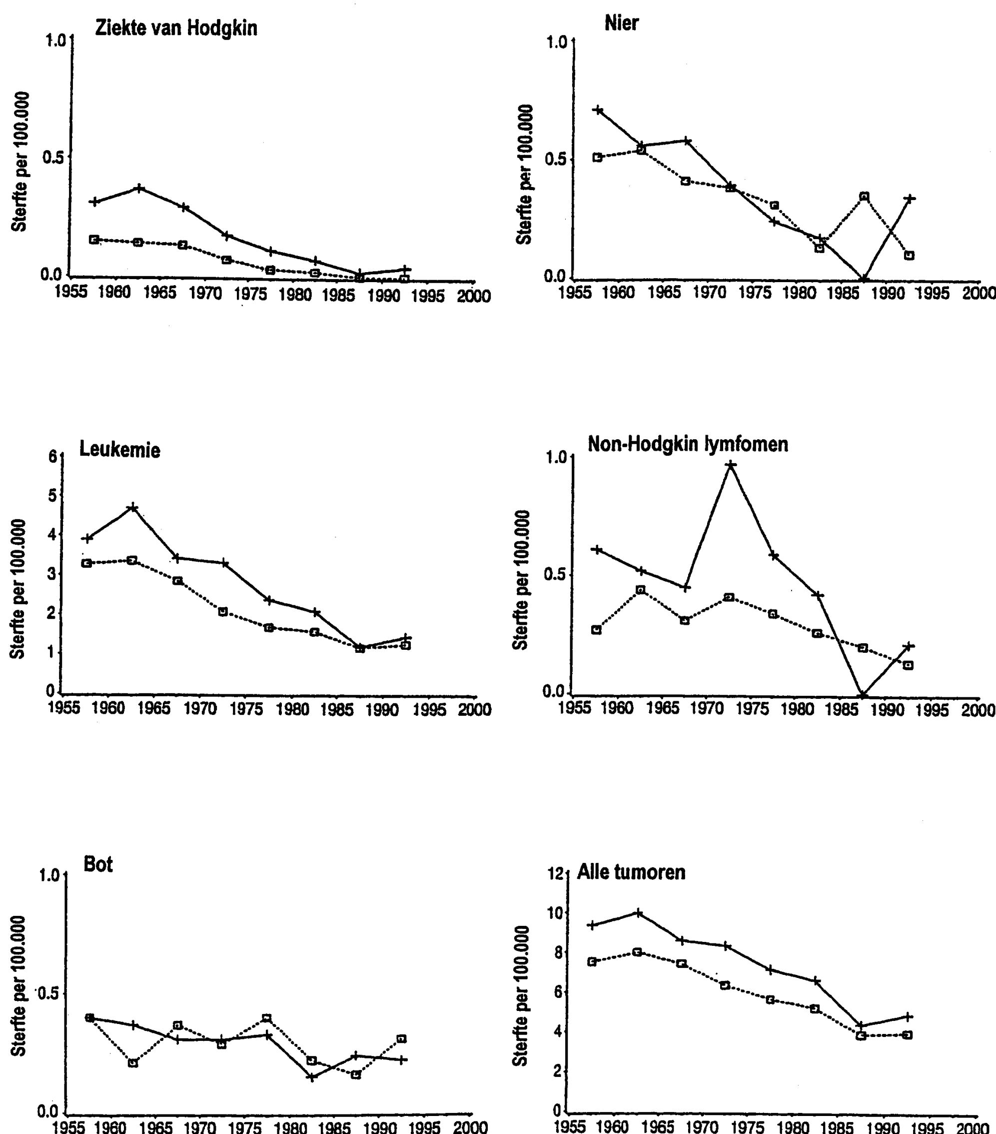
Le troisième chapitre consiste en deux études prospectives. Nous démontrons d'abord avec la technique APAAP et les anticorps monoclonaux C219 et 4E3 que l'expression de P-gp sur la membrane des cellules leucémiques est associée à un risque manifestement plus élevé de rechutes leucémiques. Les résultats de la deuxième étude confirment l'importance pronostique de P-gp chez les enfants atteints de LLA mais seulement si les résultats de l'examen immunocytochimique (anticorps monoclonaux 4E3 et MRK16) sont combinés avec ceux de l'étude cytométrique fonctionelle.

Dans le quatrième chapitre, P-gp est examiné dans un groupe d'enfants atteints de neuroblastome, ganglioneuroblastome et ganglioneurome, au moyen de l'étude immunocytochimique, des études cytométriques et de la scintigraphie au ^{99m}Tc sestamibi. L'expression immunocytochimique de P-gp n'est pas associée à un risque plus élevé d'issue fatale. En utilisant la cytométrie de flux, l'expression et l'activité de P-gp sont observées souvent et en particulier dans le groupe des tumeurs localisées, histologiquement bien différenciées, et après un traitement chimiothérapique. Les résultats de la scintigraphie au ^{99m}Tc sestamibi correspondent avec ceux des études cytométriques. Par conséquent, la scintigraphie au ^{99m}Tc sestamibi n'est pas utile, ni pour le diagnostic, ni pour la prédiction de la résistance aux anticancéreux.

Contrairement à la situation observée dans la LLA, il nous paraît que P-gp n'a rien à voir avec la résistance aux anticancéreux dans le neuroblastome mais qu'elle est plutôt un indice de différenciation.

INLEIDING TOT HET ONDERZOEK

In de voorbije decennia stegen wereldwijd de overlevingskansen van kinderen met kanker op spectaculaire wijze. Momenteel kunnen meer dan 70 % van de patiënten behandeld worden en definitief genezen.
Op basis van sterftecijfers afkomstig van de WHO databank beschreef Levi et al. (2001) de evolutie van de mortaliteit tussen 1955 en 1995 voor kinderen van verschillende leeftijdsgroepen (0-4, 5-9 en 10-14 jaar) uit 23 Europese landen. Zowel de globale mortaliteit als deze ten gevolge van leukemie, lymfomen of tumoren van het bot, de nier en het oog werden besproken. In figuur 1 wordt de evolutie van de overlevingskansen voor Belgische kinderen met verschillende tumoren geïllustreerd.



Figuur 1: Evolutie van mortaliteitscijfers ten gevolge van enkele maligniteiten (gestandaardiseerd voor leeftijd, 0-14 jaar) in België; 1955-1995 (+—+: jongens; — : meisjes).

In de West-Europese landen daalde de mortaliteit voor leukemie en enkele andere maligniteiten (Hodgkin lymfomen, Non-Hodgkin lymfomen en tumoren afkomstig van het bot) vanaf het midden van de jaren 60 met gemiddeld 60 %, en dit voor zowel jongens als meisjes. Deze daling werd toegeschreven aan de ontwikkeling van doeltreffende behandelingsschema's, het toepassen van goede ondersteunende maatregelen en betere diagnostische technieken.

Een soortgelijke verbetering van de overleving van Europese kinderen met acute lymfoblasten leukemie (ALL) werd recent ook vastgesteld in de "EUROCARE" studie (Coebergh et al., 2001).
In deze studie werden 13 344 kinderen afkomstig uit 17 Europese landen (België niet inbegrepen) bestudeerd. Men berekende via een Cox regressie analyse dat de 5-jaars overleving tussen 1978 en 1992 in belangrijke mate toenam. Het relatieve risico ("hazard ratio") op overlijden t.o.v. de periode 1978-1981 bedroeg 0.72 tussen 1982 en 1985, 0.59 tussen 1986 en 1989, en 0.49 in de periode 1990-1992.

De lange-termijn EFS bij kinderen met ALL, behandeld volgens ondermeer de huidige EORTC-CLCG "trials", bedraagt momenteel bijna 80 %. Door de heterogeniteit binnen de acute lymfatische leukemieën zelf, hebben sommige patiënten met ALL een slechtere prognose. Daarom worden de kinderen bij diagnose aan de hand van een aantal prognostische kenmerken ingedeeld in verschillende subgroepen die een aangepaste behandeling krijgen. De kinderen met een hoog risico krijgen een zwaardere behandeling dan de standaardgroep. Bovendien wordt, om de nadelige effecten van chemotherapie tot een minimum te beperken, aan kinderen in de laag-risicogroep een lichtere behandeling gegeven, zonder hierbij de overlevingskansen te compromitteren (Pui et al., 1998).
De gekende prognostische factoren bij ALL op kinderleeftijd worden meer in detail besproken in de algemene inleiding.

De evolutie van de overlevingskansen voor Europese kinderen met een neuroblastoom werd ook onderzocht aan de hand van gegevens uit de EUROCARE II studie (Spix et al., 2001). Er werden 1094 kinderen tussen 0 en 14 jaar afkomstig uit 16 landen (geen Belgische gegevens beschikbaar) bestudeerd. Men zag voor de totale groep patiënten een daling van het sterfterisico van 37 % tussen de periodes 1978-1981 en 1990-1992. Bij zuigelingen was er een betere 5-jaars overleving (79 %) in vergelijking met oudere kinderen (30-33 %). De 5-jaars overleving was ook zeer verschillend van land tot land, en dit vermoedelijk als gevolg van de sterk uiteenlopende aantallen geregistreerde zuigelingen. De prognose van een neuroblastoompatiënt wordt echter niet alleen beïnvloed door de leeftijd van de patiënt, maar ook door het stadium van de ziekte en verschillende biologische parameters zoals *MYCN* amplificatie. Deze prognostische factoren worden in deze studie echter niet besproken.
De 5-jaars overleving van de kinderen ouder dan één jaar met een stadium 4 neuroblastoom of met een tumor met *MYCN* oncogen amplificatie is toegenomen dankzij zeer intensieve behandelingsschema's, maar stijgt toch nauwelijks boven 30 % (Berthold en Hero, 2000; Matthay et al., 1999; Ladenstein et al., 1998).

Ondanks de identificatie van talrijke prognostische factoren, zowel bij ALL als bij het neuroblastoom, en de toepassing van een behandeling aangepast aan deze risicofactoren, zijn er een aantal kinderen met een slechte prognose door het ontwikkelen van resistentie

aan de toegediende cytostatica. Precies om ook deze kinderen doeltreffend te kunnen behandelen moet de rol van resistentiemechanismen zoals de expressie van het P-glycoproteïne (P-gp) nagekeken worden.

DOELSTELLINGEN VAN HET ONDERZOEK

De beschikbare literatuurgegevens over het prognostisch belang van P-gp, zowel bij ALL op kinderleeftijd als bij neuroblastaire tumoren zijn niet eenduidig.

P-gp wordt in uiteenlopende percentages leukemische cellen teruggevonden, afhankelijk van de toegepaste detectietechniek (cfr. Hoofdstuk 2 en 3). Er werden reeds belangrijke prospectieve studies bij kinderen met ALL uitgevoerd, waarin P-gp al dan niet bruikbaar bleek als parameter om leukemisch recidief te voorspellen (Goasguen et al., 1993; Dhooge et al., 1999; Wuchter et al., 2000; Kanerva et al., 2001). Deze studies zijn onderling moeilijk vergelijkbaar omdat ze gebaseerd zijn op andere detectiemethodes. Er wordt gebruik gemaakt van verschillende monoklonale antilichamen (MoAbs), verschillende “cut-off” waarden voor het vaststellen van positiviteit, enz. Internationale werkgroepen bevelen daarom aan om meerdere complementaire gestandaardiseerde technieken te gebruiken (Beck et al., 1996; Marie et al., 1997).
In dit proefschrift worden verschillende immunologische testen (immuuncytochemische en flowcytometrische) toegepast waarbij meerdere MoAbs gebruikt worden. Deze immunologische testen worden vergeleken met een functionele flowcytometrische test, die als gouden standaard beschouwd wordt. We willen bovendien het prognostisch belang van P-gp bij ALL op kinderleeftijd bevestigen, zowel immuuncytochemisch als via een combinatie van verschillende technieken.

Goldie en Coldman (1984) veronderstelden dat tumorcellen resistentie verwerven door spontane mutaties of door adaptatie. De resistente klonen zijn aanvankelijk nauwelijks te detecteren, maar zullen uiteindelijk door selectie en groeivoordeel overheersen.
In dit onderzoek zal nagekeken worden of P-gp expressie reeds intrinsiek aanwezig is in een leukemische cel of verworven wordt na toediening van chemotherapie aan de patiënt.

Dit onderzoek beoogt ook meer inzicht te geven in de prognostische waarde van P-gp bij neuroblastaire tumoren of subgroepen hiervan op kinderleeftijd. De besluiten van vorige studies zijn zeer uiteenlopend en worden besproken in een literatuuroverzicht (cfr. Hoofdstuk 1). Meestal werd MDR1 expressie nagegaan via northern blot, RNA slot blot (Goldstein et al., 1989; Bourhis et al., 1989; Bénard et al., 1994) of (RT-)PCR (Haber et al., 1997; Oda et al., 1997). Bij deze technieken wordt “bulk”materiaal gebruikt waardoor de specificiteit van de testen niet optimaal is. P-gp expressie werd ook nagekeken met verschillende immuuncyto- of histochemische technieken met verschillende MoAbs. Favrot et al. (1991) en O’Meara et al. (1992) pasten klassieke immuuncytochemische protocols toe en konden nooit P-gp expressie terugvinden. Andere onderzoekers analyseerden neuroblastoomstalen met immuuncytochemische testen die aangepast werden om een sterke gevoeligheid te bekomen. Zij toonden P-gp expressie aan maar beschreven uiteenlopende klinische implicaties van deze positiviteit (Chan et al., 1991; Dhooge et al., 1997). Bovendien beschreef Bates et al. in 1989 een experiment waarin de expressie van niet-functioneel P-gp geïnduceerd werd in een neuroblastoomcellijn behandeld met retinoïnezuur.
We vermoeden dat de controverse rond het belang van P-gp in neuroblastomen beïnvloed wordt door een dissociatie van expressie en functie. Daarom behandelden we stalen van neuroblastomen zodat ze ook flowcytometrisch geanalyseerd konden worden en op die manier ook de functie van P-gp in vitro nagekeken kon worden.

Bovendien pasten we ook beeldvorming met Technetium-99m sestamibi (^{99m}Tc sestamibi) toe. Dit isotoop is een substraat van P-gp en is dus bruikbaar om de activiteit van P-gp in vivo na te kijken in primaire tumoren en in metastasen. Als aangetoond kan worden dat P-gp een belangrijke prognostische parameter is in (bepaalde subgroepen van) neuroblastomen, kan dit isotopenonderzoek een nuttig middel zijn om MDR te bepalen bij elke patiënt, zowel voor het starten van de behandeling als tijdens de behandeling (Hendrikse et al., 1999).

Tenslotte wordt in dit onderzoek ook nagekeken in welke mate P-gp expressie en/of functie in vitro en in vivo terug te vinden zijn bij gedifferentieerde neuroblastaire tumoren, namelijk ganglioneuromen en ganglioneuroblastomen.

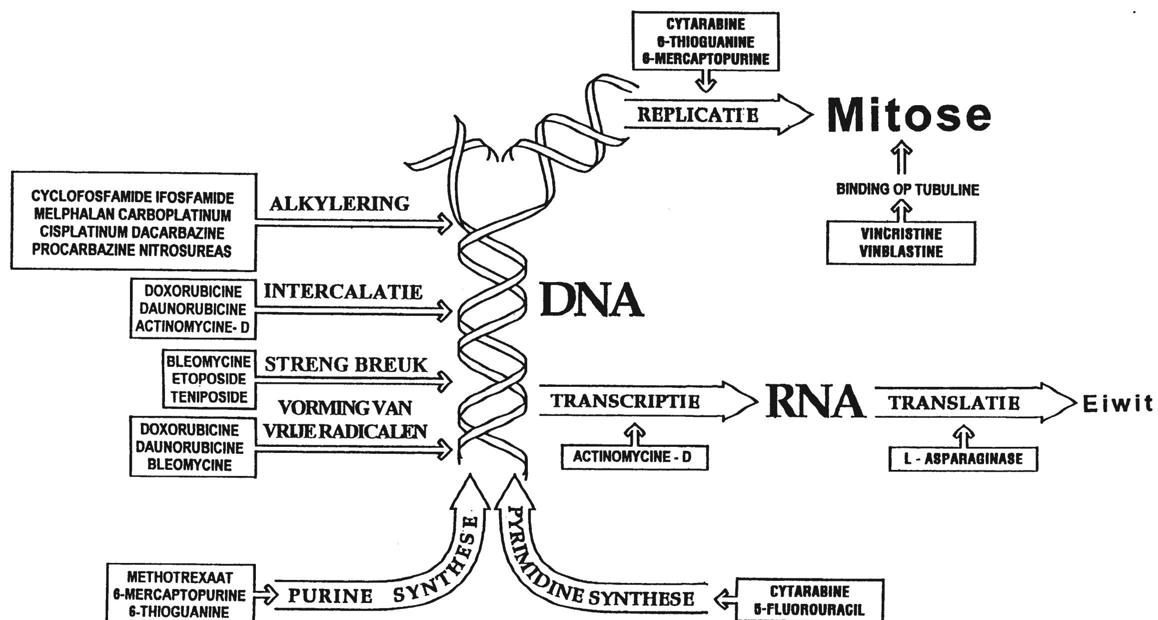
HOOFDSTUK 1

ALGEMENE INLEIDING

1 Chemotherapieresistentie

1.1 Chemotherapie en het ontstaan van resistentie

Chemotherapie is een fundamenteel onderdeel van de behandeling van maligne aandoeningen op kinderleeftijd.
Het cytotoxisch effect van veelgebruikte chemotherapeutische middelen berust op een interferentie met de synthese of de functie van DNA. Dit wordt schematisch voorgesteld in figuur 2. De DNA-schade die ontstaat door de inwerking van het cytostaticum resulteert in necrose of in geprogrammeerde celdood (Riccardi et al., 1998).



Figuur 2: Aangrijpingsplaats van frequent gebruikte cytostatica in de pediatrische oncologie.

De werkingsmechanismen van cytostatica zijn uiteenlopend (overzicht in Baltzer Cleri en Berkery-Haywood, 1999). Soms is er een selectieve werking in één bepaalde fase van de celcyclus. Dit is bijvoorbeeld het geval voor vinca-alkaloïden, epipodofyllotoxinen en antimetabolieten. Andere cytostatica (zoals actinomycine-D, cisplatinum en bepaalde alkylerende produkten) zijn niet specifiek afhankelijk van een bepaalde fase van de celcyclus.

Bepaalde cytostatica hebben meerdere aangrijpingspunten. De antracyclinen doxorubicine en daunorubicine zijn hiervan voorbeelden. Enerzijds kunnen ze ingebouwd worden tussen de DNA-baseparen waardoor ze interfereren met een correcte DNA-synthese. Anderzijds zijn ze ook verantwoordelijk voor de vorming van vrije radicalen en de inhibitie van het enzym DNA topoisomerase II. Bleomycine en de epipodofyllotoxinen etoposide en teniposide inhiberen ook de werking van het topoisomerase II waardoor breuken in de DNA-strengen niet hersteld kunnen worden. Actinomycine-D kan net zoals doxorubicine en daunorubicine tussen de DNA-strengen ingebouwd worden. Dit resulteert in een inhibitie van de replicatie van DNA en van de RNA- en eiwitsynthese.

De alkylerende producten veroorzaken breuken en "cross-linking" van de DNA-strengen waardoor een correcte replicatie of transcriptie verhinderd wordt. "Cross-links" binnenin en tussen DNA-strengen worden eveneens veroorzaakt door de zware metalen cisplatinum en carboplatinum. Antimetabolieten zoals 6-mercaptopurine, 6-thioguanine, methotrexaat en cytarabine beïnvloeden de synthese van DNA en RNA. Methotrexaat blokkeert de werking van het enzym dihydrofolaatreductase (DHFR) waardoor foliumzuur niet wordt omgezet naar tetrahydrofoliumzuur. Hierdoor worden DNA-, RNA- en eiwitsynthese geïnhibeerd. De vinca-alkaloïden vincristine en vinblastine binden daarentegen op het tubuline van de mitotische spoelfiguur waardoor de mitose onderbroken wordt. Asparaginase is een enzym dat het aminozuur asparagine hydrolyseert waardoor de eiwitsynthese stopt.

De efficiëntie van chemotherapie wordt door vele farmacologische en tumorale factoren bepaald zoals de toegediende dosis van het cytostaticum, de opname en metabolisatie van het cytostaticum, de localisatie, grootte en vascularisatie van de tumor, het aantal actief delende cellen in de tumor, ...

Een belangrijke oorzaak van het falen van chemotherapie is een resistentie van de tumorcel zelf. Zo kunnen tumorcellen al vanaf het begin resistent zijn voor de toegediende chemotherapeutica (primaire of intrinsieke resistentie). Soms worden ze pas resistent gedurende of na de behandeling. Bij hematologische maligniteiten en bij neuroblastomen wordt vooral deze "verworven" vorm van resistentie teruggevonden (Verhoef et al., 1995; De Moerloose et al., 1999; Berthold et al., 2000).

Een biologische verklaring voor deze resistentie werd beschreven door Goldie en Coldman (1979, 1984). Volgens hun theorieën kunnen resistente cellen reeds bij diagnose aanwezig zijn ofwel ontstaan door mutaties. Deze resistente klonen zullen vervolgens door selectie preferentieel in aantal toenemen en overleven in het toxisch milieu.

Het resistentiepatroon van een cel kan gericht zijn tegen één cytostaticum. Voorbeelden hiervan zijn de resistentie aan methotrexaat door verminderde activiteit of verminderde transportsnelheid van de "folaatcarrier" of door een overproduktie van het DHFR; de afwezigheid van het enzym deoxycytidinekinase waardoor cytarabine niet meer wordt omgezet tot het actieve aracytidine-trifosfaat; een verhoogde concentratie van metallothioneïne dat cisplatinum en enkele alkylerende produkten kan binden en hierdoor inactiveert; een verlaagd gehalte cytochroom P450 waardoor bepaalde cytostatica zoals cisplatinum of cyclofosfamide niet geactiveerd kunnen worden; de inductie van asparaginesynthetase waardoor tumorcellen hun eigen asparagine kunnen aanmaken enz. Resistentie kan ook ontstaan door een versneld herstel van DNA-schade veroorzaakt door chemotherapie (Borst et al., 1991; Harrison et al., 1995).

In 1970 werd door Biedler voor het eerst vastgesteld dat een cellijn die in contact gebracht was met actinomycine-D ook resistent werd voor daunorubicine en vinblastine. Deze cellulaire resistentie tegen meerdere structureel en functioneel niet-verwante cytostatica die ontstaat na blootstelling aan slechts één van deze cytostatica, wordt multidrugresistentie (MDR) genoemd.

MDR kan veroorzaakt worden door verschillende mechanismen (review door Harrison et al., 1995 en door Van den Heuvel-Eibrinck et al., 2000):

1. Toegenomen expulsie van cytostatica uit de resistente cel:

Expressie van P-gp:

Het MDR1 genproduct P-gp is een glycoproteïne gelegen in de celmembraan dat in 1979 door Riordan en Ling geïsoleerd werd uit multidrugresistente cellijnen. Als lid van de familie ATP-bindingscassette (ABC) eiwitten, fungeert het als een ATP-afhankelijke "pomp" die verschillende substraten (waaronder ook bepaalde cytostatica) vanuit het cytosol van de cel naar buiten transporteert. De structuur, de functie en de klinische relevantie van P-gp worden besproken in deel 1.2.

Expressie van het multidrugresistentie-geassocieerd proteïne (MRP):

Een ander lid van de ABC-transporter superfamilie, het multidrugresistentie-geassocieerd proteïne (MRP), werd in 1992 voor het eerst teruggevonden in cellijnen met een MDR-fenotype maar zonder P-gp expressie (Cole et al., 1992). Sindsdien werden minstens zeven leden van de MRP-familie gekarakteriseerd (MRP1, MRP2 of c-MOAT, en MRP3-7) (Kool et al., 1997; Borst et al., 2000; Litman et al., 2001).

Het *MRP1* gen is gelocaliseerd op chromosoom 16p13.1. Het codeert voor een eiwit van 1531 aminozuren met een moleculair gewicht van 190 kDa. Er is 15 % homologie tussen de aminozuren van MRP1 en P-gp. In gentransfectie-experimenten werd aangetoond dat overexpressie van MRP1 resulteerde in verlaagde intracellulaire concentraties van bepaalde cytostatica, zoals ondermeer antracyclinen.

MRP1 komt in bijna alle menselijke weefsels voor. Het wordt niet enkel in de celmembraan teruggevonden, maar ook in het endoplasmatisch reticulum, Golgi complex en intracellulaire vesikels (Hipfner et al., 1999).

Muizen zonder MRP1, een leukotriëne C4 "transporter", vertonen enkel een verminderd antwoord op inflammatoire stimuli en zijn zeer gevoelig aan etoposide. MRP2 zorgt voor secretie van bilirubineglucuronide van de lever naar de gal. Mensen zonder MRP2 vertonen het Dubin-Johnson syndroom (Paulusma et al., 1997).
De fysiologische rol van de andere MRPs is nog grotendeels onbekend. Een overzicht van recent onderzoek naar de structuur, de fysiologische functie, de substraatspecifiteit en de inhibitoren van de MRP-eiwitten wordt beschreven door Borst et al. (2000). In vitro experimenten toonden aan dat de MRPs organische anionen (zoals methotrexaat) en neutrale produkten geconjugeerd met glutathion, glucuronaat of sulfaat kunnen transporteren. Ook transport van niet-geconjugeerde cytostatica samen met glutathion is beschreven (van der Kolk et al., 1999). MRP1 verwijdert antracyclinen zoals doxorubicine, daunorubicine en idarubicine, mitoxantrone en etoposide uit de cel.
De substraat specificiteit van MRP1 en P-gp is niet volledig identisch. Zo zal MRP1 bijvoorbeeld nauwelijks paclitaxel transporteren en de affiniteit van MRP1 voor vincristine is laag. P-gp heeft daarentegen een lage affiniteit voor anionen zoals methotrexaat. Gentransfectie-experimenten toonden aan dat MRP2 resistentie kan

veroorzaken tegen cisplatinum geconjugeerd met glutathion. Verder is er een overeenkomst tussen de substraatspecificiteit van MRP2 en deze van MRP1. In vitro experimenten met MRP3 toonden resistentie aan voor methotrexaat, etoposide en teniposide. MRP4 overexpressie is geassocieerd met een sterke resistentie tegen bepaalde nucleotide-analogen (zoals azidothymidine). Of de MRPs ook resistentie kunnen veroorzaken tegen andere antimetabolieten zoals 6-mercaptopurine en thioguanine, wordt nog onderzocht.

Net zoals voor P-gp, probeert men ook inhibitoren voor MRP-eiwitten te ontwikkelen. De leukotriëne antagonist MK571, genisteïne en probenecid kunnen in vitro de functie van MRP1 inhiberen, maar zijn niet toepasbaar in de kliniek (Borst et al., 2000; Sonneveld et al., 2000).

Expressie van andere ABC-eiwitten:

→ **sP-gp** (sister of P-glycoprotein):
De aminozuursequentie van dit eiwit is voor 61 % homoloog met deze van P-gp. Vermoedelijk is sP-gp een produkt van een vroege genduplicatie. Men kon het, tot op heden, nog niet terugvinden in menselijke tumoren (Childs et al., 1995).

→ **TAP** (transporter associated with antigen-presentation):
Dit eiwit is samengesteld uit het TAP1- en het TAP2-eiwit. Het zorgt voor de verplaatsing van peptiden van het cytosol naar het endoplasmatisch reticulum, waar deze nadien gekoppeld worden aan moleculen van het majeur histocompatibiliteitscomplex klasse I, en naar de celmembraan gebracht worden voor presentatie aan cytotoxische T-lymfocyten (Neefjes et al., 1993). TAP werd ook teruggevonden in resistente tumorcellijnen die geen P-gp expressie vertonen. Via gentransfectie-experimenten kon men aantonen dat TAP cellen resistent maakt aan doxorubicine (Izquierdo et al., 1996). Men vermoedt dat deze resistentie ontstaat door een sequestratie van antracyclinen in het endoplasmatisch reticulum.

→ **ARA** (Anthracycline resistance-associated protein):
Dit eiwit van 49.5 kDa lijkt op het C-terminaal gedeelte van MRP6 (Longhurst et al., 1996). Het 3' uiteinde van het *MRP6* gen is identisch aan het 3' uiteinde van het *ARA* gen. Men vermoedt dan ook dat ARA een "splice" variant van MRP6 is. Aangezien het *MRP6* gen (en dus ook het *ARA* gen) gelocaliseerd is naast het *MRP1* gen op chromosoom 16 worden beide gecoamplificeerd met *MRP1* (Kool et al., 1997).

→ **BCRP** (breast cancer resistance protein, P-95, MXR, ABCP, ABCG2):
BCRP, een eiwit van 95 kDa, werd reeds in 1990 teruggevonden in de celmembraan van een doxorubicine-resistente kloon van de MCF-7 borstkankercellijn (Chen et al., 1990). Deze resistente cellijn vertoont geen P-gp expressie. Ze is sterk resistent aan antracyclinen, melphalan en teniposide, maar niet aan vinblastine. Men noemt BCRP ook een "half-transporter" omdat BCRP een homodimeer of een heterodimeer moet vormen om een actieve pomp te worden. BCRP werd ook teruggevonden in klinische stalen van patiënten met een vaste tumor of leukemie refractair aan doxorubicine (Doyle et al., 1993 en 1996; Ross et al., 2000a en 2000b).

Expressie van het "lung resistance protein" LRP:

In 1993 werd LRP ontdekt in een antracycline-resistente cellijn die geen P-gp expressie vertoonde (Scheper et al., 1993). Het LRP, ook "major vault protein" genoemd, is een eiwit van 110 kD. Het wordt gecodeerd door een gen gelegen op de korte arm van chromosoom 16 (16p13.2). Het is een bestanddeel van intracellulaire organellen die opgebouwd zijn uit ribonucleoproteïnen ("vaults" geheten) en die instaan voor het transport van produkten tussen de kern en het cytoplasma en eveneens voor de exocytose ervan. Bij immuunhistochemische kleuringen vindt men 5 % van het LRP terug in de kernmembraan; 95 % bevindt zich in cytoplasmatische vesikels (Wiemer et al., 1998). Men vermoedt dat LRP een rol speelt in detoxificatieprocessen. In vitro veroorzaakt LRP ondermeer resistentie tegen antracyclinen, vincristine, cisplatinum, carboplatinum en alkylerende produkten zoals melphalan (Scheper et al., 1996; Raaijmakers et al., 1998).

2. <u>Versnelde intracellulaire detoxificatie via het glutathionmetabolisme:</u>

Glutathion-S-transferasen (GST) zijn enzymen die betrokken zijn in de detoxificatie van een aantal produkten, waaronder ook cytostatica. De GST worden onderverdeeld in 4 subklassen: α, μ, π en θ. Bij overexpressie van GST is er een verhoogde conjugatie van bepaalde cytostatica met glutathion, waardoor het transport, de metabolisatie en de excretie ervan toenemen. Vooral GSTπ wordt teruggevonden in tumoren (Tew et al., 1994). Dit mechanisme is vooral actief bij verworven resistentie tegen alkylerende produkten en antracyclinen zoals doxorubicine.
Recent werd aangetoond dat het transport van glutathionconjugaten uit de cel gebeurt via MRP1 en MRP2. Ook werd een inductie van zowel MRP1 als enzymes van de "glutathion-pathway" geobserveerd in tumorale cellen die in vitro aan cytostatica blootgesteld werden (overzicht door van der Kolk et al., 1999).
Ethacrynezuur wordt gebuikt in klinische studies om GSTπ te inhiberen en aldus drugresistentie te verminderen. BSO (buthionine sulphoximine) inhibeert de glutathionsynthese en verhoogt de toxiciteit van o.a. cyclofosfamide, melphalan en bleomycine (Harrison et al., 1995).

3. <u>Verminderde activiteit van het enzym topoisomerase II:</u>

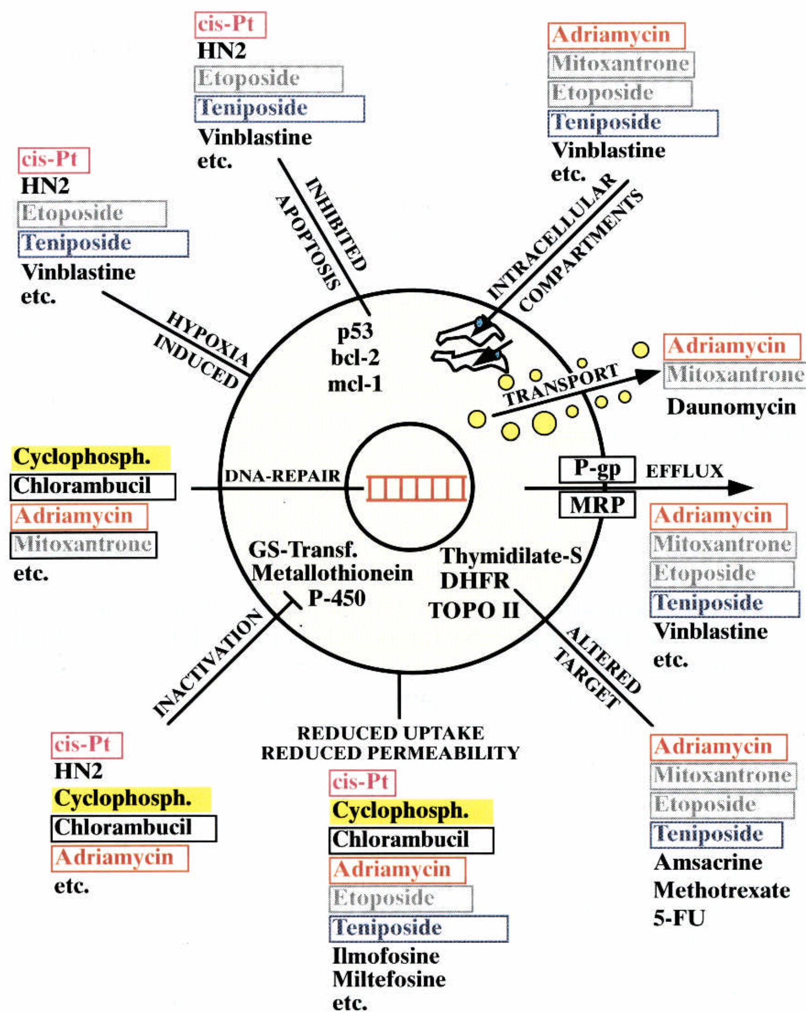
Topoisomerase II zorgt tijdens de celdeling van zowel de normale cel als de kankercel voor de aanmaak van "duplex breaks" in het DNA. Het katalyseert de uiteinden van de breuken en herstelt deze na verdubbeling van het DNA. Een aantal cytostatica, zoals de antracyclinen, acridines, mitoxantronederivaten, ellipticine en epipodofyllotoxines, blokkeren de werking van topoisomerase II, wat uiteindelijk leidt tot breuken in het delende DNA met celdood tot gevolg. Delende maligne cellen kunnen resistentie verwerven door verlaging van de topoisomerase II-concentratie of door inductie van mutante (resistente) vormen van topoisomerase II (Borst, 1991, Alton et al., 1993). Of topoisomerase II-gemedieerde resistentie ook klinisch belang heeft is nog niet opgehelderd (Filipits et al., 1996).

4. Verminderde apoptose:

Inhibitie van een door cytostatica geïnduceerde apoptose kan ontstaan door afwezigheid van het tumorsuppressorgen *p53*; door overexpressie van oncogenen zoals *bcl2* of door veranderingen in de CD95/FAS "pathway". Afwezigheid van het tumorsuppressorgen *p53* kan bijvoorbeeld resistentie tegen etoposide geven (Lowe et al., 1993); overexpressie van het *bcl2* oncogen veroorzaakt ondermeer resistentie in lymfoomcellijnen (Miyashita et al., 1993).

Er kunnen meerdere resistentiemechanismen voorkomen in eenzelfde tumorcel (schematisch voorgesteld in figuur 3). Ook zal de aanwezigheid van bepaalde resistentiemechanismen sterk variëren in verschillende subpopulaties van een tumormassa. Vooral in grote tumoren is er een sterke cellulaire heterogeniteit en bestaat er een belangrijke kans op resistente klonen (Borst, 1991).

CELLULAIRE RESISTENTIEMECHANISMEN



Figuur 3: Overzicht van cellulaire resistentiemechanismen. P-gp: P-glycoproteïne; MRP: multidrugresistentie-geassocieerd proteïne; TOPO II: topoisomerase II; DHFR: dihydrofolaat reductase: GS-Transf: glutathion-S-transferase. (H. Grunicke en J. Hofman (Innsbruck))

1.2 Chemotherapieresistentie en P-glycoproteïne

P-GLYCOPROTEÏNE IN DE AANPAK VAN CHEMOTHERAPIERESISTENTIE

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Tijdschrift voor Geneeskunde 1999; 55: 655-660

Tijdschr. voor Geneeskunde, **55**, nr. 9, 1999

OVERZICHT

P-GLYCOPROTEÏNE IN DE AANPAK VAN CHEMOTHERAPIERESISTENTIE

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Samenvatting

In dit artikel wordt een overzicht gegeven van multidrugresistentie (MDR), een verschijnsel verantwoordelijk voor het falen van chemotherapie.

De expressie van P-glycoproteïne (P-gp) is een van de best gekende mechanismen die ertoe aanleiding geven. P-gp is structureel homoloog aan bacteriële transporteiwitten. Zij wordt teruggevonden in sommige normale weefsels en in bepaalde tumoren.

De structuur, functie en klinische relevantie van P-gp worden uitgelegd, evenals de mogelijkheden om, rekening houdend met de P-gp-status van de tumorcellen, een optimaal antwoord op chemotherapie te verkrijgen.

Inleiding

„Drug resistance follows the development of new drugs like a faithful shadow". Deze uitspraak van Paul Ehrlich, een bacterioloog uit de vorige eeuw, blijft actueel. Iedere cel van om het even welk organisme is in staat om resistent te worden tegen toxische stoffen. Bij de behandeling van maligne aandoeningen, ook na een initieel goede reactie op de ingestelde behandeling, wordt men frequent geconfronteerd met een ontoereikend of zelfs afwezig antwoord op chemotherapie als gevolg van het optreden van cellulaire resistentie (tabel 1). De capaciteit van tumorcellen om resistent te worden tegen één cytostaticum, bijvoorbeeld methotrexaat, was min of meer verwacht; het resistentiepatroon tegen combinaties van meerdere cytostatica daarentegen was een verrassende observatie. Deze laatste vorm van resistentie wordt multidrugresistentie (MDR) genoemd (1, 2).

Het MDR-fenotype werd voor het eerst beschreven in 1970 (3). Meerdere modellen voor het ontstaan ervan werden vooropgesteld: ofwel komen reeds vóór het starten van de behandeling resistente cellen voor in de heterogene tumor, en zullen bij voorkeur deze in aantal toenemen naarmate de gevoelige cellen afsterven; ofwel ontwikkelen oorspronkelijk gevoelige cellen resistentiepatronen door blootstelling aan de cytotoxische medicatie. Ook de combinatie van deze twee mechanismen zal tot klinische MDR leiden.

De oorzaken van MDR zijn meervoudig en onderling niet exclusief (tabel 1). Het best bestudeerde van deze mechanismen is de expressie van P-glycoproteïne (P-gp).

P-gp als oorzaak van MDR

P-gp werd in 1979 geïsoleerd door Riordan en Ling uit multidrugresistente cellijnen. Ze gaven het de naam „P"-glycoproteïne omdat ze dachten dat het aan de cel een verhoogde „permeabiliteit" gaf (4).

Nadien bleek dat in de resistente cellen een energieafhankelijk mechanisme functioneerde dat cystostatica actief uit de cel verwijderde. Dit werd geobserveerd in meerdere cellijnen, afkomstig van zowel knaagdieren als van de mens, die resistent waren gemaakt tegen adriamycine, colchicine, daunorubicine, vinblastine of vincristine. Steeds werd een vermin-

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TABEL 1

Bepalende factoren van het antwoord op chemotherapie

1. Farmacokinetica/Farmacodynamica van het cytostaticum
2. Biologische factoren
   - Tumorgrootte
   - Vascularisatie
   - Oxygenatie
   - Immuuncompetentie
3. Cellulaire resistentie
   - Multidrugresistentie (MDR)
     - expressie van MDR1 (P-glycoproteïne)
     - expressie van MRP (Multidrug Resistance associated Protein)
     - expressie van LRP (Lung Resistance Protein)
     - verminderde activiteit van topoïsomerase II
     - detoxificatie via het glutathionmetabolisme
     - toename in herstelmechanismen van het DNA
   - Resistentie tegen cisplatine
   - Resistentie tegen methotrexaat
   - Andere

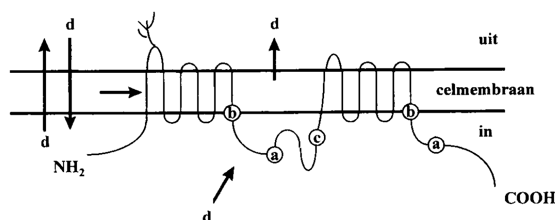

Fig. 1: Structuur van P-glycoproteïne.
P-gp bestaat uit twee helften met elk zes transmembranaire segmenten en één ATP-bindingsplaats (**a**). De andere drie bindingsplaatsen op P-gp zijn twee vinblastine/verapamil/ciclosporine-A-bindingsplaatsen (**b**) en één bindingsplaats voor andere calciumkanaalblokkeerders (**c**). De pijlen stellen het transport van P-gp-substraten (**d**) voor.

derde intracellulaire ophoping van deze cytostatica vastgesteld, alsook veranderingen in de plasmamembraan van de cel, namelijk expressie van P-gp.

Verschillende onderzoekers bewezen via gentransfectie-experimenten, hybridisatietechnieken en het gebruik van monoklonale antilichamen dat P-gp verantwoordelijk was voor het MDR-fenotype in de bestudeerde cellijnen.

De voorstelling van P-gp die door Kartner en Ling (5) werd vooropgesteld aan de hand van een analyse van cDNA, is nog steeds hypothetisch. De transmembranaire topologie van P-gp en de plaatsen waar substraatherkenning en ATP-hydrolyse gebeuren, werden recentelijk gekarakteriseerd (6) (fig. 1).

De P-glycoproteïnen zijn glycoproteïnen van ongeveer 170 kD groot, gelegen in de plasmacelmembraan. Ze behoren tot de familie ATP-bindingscassette(ABC)-transporteiwitten en komen voor in kankercellen maar ook in veel normale cellen.

Bij de mens zijn twee isovormen beschreven, MDR1 en MDR3. Dit zijn twee verschillende eiwitten; hun coderende genen liggen beide op chromosoom 7, echter op verschillende loci. MDR3 heeft niets te maken met het MDR-fenotype, maar staat in voor de excretie van fosfatidylcholine en fosfolipiden van de hepatocyt naar de gal (7). MDR1 daarentegen geeft aanleiding tot de typische vorm van MDR.

Men definieert deze typische „klassieke" vorm van MDR als een cellulaire resistentie tegen een groep (grote, hydrofobe) cytostatica die géén gemeenschappelijke structuur noch functie hebben (tabel 2). Deze resistentie kan ontstaan na blootstelling van de cellen aan slechts één cytostaticum en is te wijten aan een verhoogde uitstoting van de cytostatica uit de resistente cel.

Hoe P-gp precies werkt is nog steeds niet opgehelderd. Klassiek beschrijft men P-gp als een pomp die substraten vanuit het cytoplasma naar extracellulair pompt. In de meest recente theorie wordt geopperd dat P-gp, als een „flippase", substraten van in het binnenblad naar het buitenblad van de plasmamembraan wipt (8).

TABEL 2

P-gp-substraten

Antimitotica:	vincristine, vinblastine, colchicine (*sterke resistentie*)
Antracyclinen:	doxorubicine, daunorubicine (*intermediaire resistentie*), dihydroxyantracenedion, mitoxantron
Antibiotica:	actinomycine D (*sterke resistentie*), mitramycine, puromycine
Epipodofyllotoxinen:	etoposide, tenoposide (*intermediaire resistentie*)
Andere:	emetine, mitomycine, gramicidine D, ethidiumbromide, taxanen, cytochalasine B, topotecan

Voorkomen en functie van P-gp in normale weefsels

P-gp is een zeer geconserveerde molecule aangezien ze niet alleen kan teruggevonden worden bij de mens maar ook bij verschillende dieren.

De structuur van P-gp is homoloog aan bacteriële transporteiwitten (bijvoorbeeld het hemolysine B dat bij bepaalde bacteriën instaat voor het transport van α-hemolysine uit de cel) en andere eiwitten van het ABC-systeem. Ook het „cystic fibrosis transmembrane regulator"(CFTR)-eiwit is homoloog aan P-gp.

P-gp wordt aangetroffen in normale menselijke weefsels (9): in de apicale membraan van de epitheelcellen van het colon, in de dunne darm, bijnier, nier, pancreas en galcanaliculi, in het placentair trofoblast, in het endotheel van de testes en van de bloed-hersenbarrière, ... Ook bepaalde T-lymfocyten en wittebloedcelprecursoren in het beenmerg vertonen P-gp-expressie (10).

De fysiologische functies van P-gp zijn nog grotendeels speculatief (7). P-gp zou ondermeer endogene of exogene toxinen kunnen elimineren door excretie in de darm, de lever of de nier. In de bijnier is transport van cortisol, corticosteron en aldosteron via P-gp aangetoond; vermoedelijk is dit transport van steroïden ook in de placenta aanwezig. P-gp kan eveneens instaan voor de extrusie van cytokinen die niet op klassieke wijze uit de cel geëxporteerd worden en lijkt ook belangrijk te zijn voor de functie van cytotoxische T- en NK-cellen, aangezien deze beïnvloed wordt door het gebruik van P-gp-remmers.

Knock-outexperimenten, recentelijk uitgevoerd bij muizen, gaven bijkomende informatie over de fysiologische functie van P-gp in verschillende weefsels (11). De afwezigheid van P-gp heeft geen effect op deze muizen, tenzij ze worden blootgesteld aan medicaties. P-gp lijkt vooral belangrijk te zijn voor de bescherming van de hersenen tegen toxische stoffen en om de opname van hydrofobe medicaties vanuit de darm te verminderen.

Dit knock-outmodel biedt eveneens de mogelijkheid om medicaties of producten te ontwikkelen met een hogere (of lagere) accumulatie ter hoogte van de hersenen of om de bijwerkingen van P-gp-remmers te bestuderen.

P-gp-expressie in tumoren

Tumoren, afkomstig van de organen waar reeds in normale omstandigheden een P-gp-expressie aanwezig is, zijn vaak intrinsiek chemoresistent. Voorbeelden van deze tumoren zijn colorectale carcinomen, niercarcinomen, hepatocellulaire carcinomen, adrenocorticaal carcinoom en pancreascarcinoom (9, 12, 13).

P-gp-expressie in borstkanker en sarcomen van de weke weefsels is minder frequent en is laag tot afwezig in longkanker, ovariumkanker, melanomen, Wilms-tumoren, prostaat-, slokdarm- en maagcarcinomen (12, 13).

In sommige tumoren, bijvoorbeeld borstkanker (14), ovariumkanker (15) en het neuroblastoom (16), kan een sterkere P-gp-expressie aangetoond worden tijdens of na de toediening van chemotherapie. Dit wordt ook wel „verworven resistentie" genoemd.

Onder de hematologische maligniteiten wordt P-gp-expressie weinig frequent teruggevonden bij acute lymfoblastenleukemie (ALL). Bij acute myeloïde leukemie (AML) van de volwassene daarentegen blijkt P-gp-expressie in ongeveer 40% van de gevallen te worden teruggevonden bij de initiële diagnose. Deze expressie is nog frequenter bij secundaire AML, AML bij bejaarden en bij recidief-AML (17). In lymfomen en multipel myelomen is de P-gp-expressie laag bij de initiële diagnose, maar vaak verhoogd bij herval na chemotherapie (18).

In meerdere studies is aangetoond dat P-gp-expressie een prognostische betekenis heeft. Dit is volgens Chan et al. het geval voor sarcomen op kinderleeftijd, waar de P-gp-expressie geassocieerd is met een slecht antwoord op chemotherapie, een verhoogd risico op recidief en een minder goede overleving (16). Baldini et al. vinden eveneens dat P-gp-expressie een belangrijke klinische parameter is voor de prognose van osteosarcoompatiënten (19). Bij AML van de volwassene is P-gp-expressie geassocieerd met een slecht antwoord op inductiechemotherapie, met een hogere recidieffrequentie en een hogere mortaliteit (20, 21).

Voor de meeste tumoren zijn echter tegenstrijdige resultaten gepubliceerd en moet de exacte waarde van P-gp als oorzaak van MDR nog worden nagekeken. Andere oorzaken van MDR blijken eveneens een prognostische waarde te hebben in bepaalde tumoren, zoals

expressie van het „multidrug resistance associated protein" (MRP) bij het neuroblastoom (22) en de expressie van het „lung resistance protein" (LRP) bij AML, ALL op kinderleeftijd, het multipel myeloom en ovariumcarcinomen (23, 24).

MDR-modulatie in vivo

De meeste pogingen om MDR te omzeilen zijn erop gebaseerd dat de pompfunctie van P-gp geïnhibeerd kan worden, waardoor de intracellulaire concentratie van het cytostaticum opnieuw toeneemt en de tumorale cel afsterft. Stoffen die P-gp inhiberen, worden ook P-gp-modulatoren genoemd (tabel 3) (25, 26).

Bij het gebruik van een P-gp-remmer verandert de farmacokinetiek van het cytostaticum, enerzijds omdat P-gp in normale weefsels (zoals lever en nier) ook geïnhibeerd wordt, en anderzijds omdat vele P-gp-modulatoren interageren met fysiologische metabole reacties waardoor cytostatica geklaard worden (27). Een combinatie van een verminderde eliminatie met een veranderde weefseldistributie heeft uiteraard als gevolg dat de dosis van het cytostaticum verlaagd moet worden om de toxiciteit ervan te verminderen. In sommige studies is het gunstig effect van een P-gp-remmer waarschijnlijk enkel te wijten aan de verhoogde concentratie van het cytostaticum, dus aan een „dosisintensificatie" (28).

Daarenboven kunnen sommige modulatoren in de praktijk moeilijk gebruikt worden, omdat ze te toxisch zijn in de concentraties waarmee P-gp-remming bekomen wordt. Zo geeft verapamil aanleiding tot hypotensie en ritmestoornissen, ciclosporine A heeft immunosuppressie en nefrotoxiciteit als belangrijke bijwerkingen.

TABEL 3

P-gp-remmers

Calciumkanaalblokkeerders (verapamil, dexverapamil, dexniguldipine)
Calmodulineremmers (trifluoperazine)
Cyclische peptiden (ciclosporine A, PSC833, SDZ 280-446)
Proteïnekinase-C-remmers (staurosporine)
Steroïden (progesteron), tamoxifen
Monoklonale antilichamen (MRK16)
Niet-cytotoxische druganalogons
Andere: cefalosporinen, chloroquine, cremofor, dipyridamol, reserpine, fenothiazine, quinine en quinidine.

Verschillende fase-I/II-klinische trials waarin P-gp-modulatoren werden toegevoegd aan de chemotherapeutische behandeling van patiënten met een hematologische maligniteit, long- of borstkanker, gaven occasioneel goede resultaten (18, 29, 30). Grote gerandomiseerde fase-III-trials, waarin de toevoeging van het ciclosporineanalogon PSC833 aan de behandeling van volwassenen met AML of multipel myeloom wordt geëvalueerd, zijn volop bezig (31).

Er bestaan ook alternatieve, meer theoretische mogelijkheden om MDR in vivo te moduleren. De toediening van cytostatica die geen substraat zijn van P-gp, zal MDR veroorzaakt door P-gp, omzeilen, maar is vaak niet mogelijk omdat tumorcellen slechts gevoelig zijn voor een beperkt gamma cytostatica.

In vitro werden biochemisch gewijzigde P-gp-substraten, die hierdoor hun cytotoxisch effect behouden maar hun affiniteit voor P-gp verliezen, uitvoerig getest. Voorbeelden hiervan zijn de conjugatie van doxorubicine aan albumine, omkapseling van doxorubicine in liposomen of een chemisch gewijzigd, fluorinebevattend doxorubicine, ME2303 (32).

P-gp kan als doeleiwit gebruikt worden voor immunotherapie. Cellen die herkend worden door het eigen immuunsysteem na binding met een monoklonaal antilichaam gericht tegen P-gp, zullen selectief vernietigd worden (33). De monoklonale antilichamen MRK16, UIC2 en HYB-241 hebben tevens een blokkerende werking op de pompfunctie van P-gp (34, 35, 36).

Ook zou gebruikgemaakt kunnen worden van „collaterale sensitiviteit". Hieronder verstaat men de verhoogde gevoeligheid van resistente cellen voor andere farmaca, bijvoorbeeld de verhoogde gevoeligheid voor steroïden of tumornecrosisfactor die waargenomen wordt bij multidrugresistente multipel-myeloomcellen (37, 38).

Besluit

Een adequate chemotherapie vereist vooreerst een goede dosering en biodisponibiliteit van de cytostatica. Het antwoord van de maligne cellen op chemotherapie zal beïnvloed

worden door biologische factoren en resistentiemechanismen. Wanneer multidrugresistentie vastgesteld wordt, kan bij de individuele patiënt geopteerd worden voor een aanpassing van het therapieschema.

Vermijden van cytostatica waartegen resistentie bestaat, gebruikmaken van collaterale sensitiviteit, inschakelen van P-gp-remmers en een minder intensieve behandeling van P-gp-negatieve patiënten zal de therapiegebonden toxiciteit doen dalen, de recidieffrequentie verminderen en uiteindelijk de overleving van de patiënt verbeteren.

De selectieve vernietiging van resistente cellen en een doeltreffende modulatie of remming van resistentiemechanismen zoals P-gp, kunnen een belangrijke stap voorwaarts betekenen in de behandeling van kanker.

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1.3 Regulatie van *MDR1* genexpressie

Het blijft nog steeds onduidelijk of het MDR fenotype ontstaat door uitselecteren en klonale expansie van vooraf bestaande resistente cellen, door inductie van MDR na blootstelling aan chemotherapie of door een combinatie van deze beide mechanismen.

Klassiek veronderstelt men dat P-gp gemedieerde MDR onstaat door *MDR1* genamplificatie, een toegenomen transcriptie of een verhoogde translatie tot het actieve eiwit.

MDR1 genamplificatie in cellijnen is soms zichtbaar als hoeveelheden extrachromosomaal of intrachromosomaal DNA (Dms (double minutes), HSRs (homogeneously staining regions) of ABRs (abnormally banding regions)). Nochtans kan deze genamplificatie niet teruggevonden worden in bepaalde cellijnen en in klinische stalen, terwijl er wel een gestegen mRNA en verhoogd eiwitgehalte aanwezig is (Biedler en Spengler, 1996).

Er zijn verschillende consensus-sequenties, verantwoordelijk voor de regulatie van gentranscriptie, beschreven in de promotorregio van het *MDR1* gen. Bovendien zijn meerdere factoren bekend die MDR1 expressie kunnen moduleren. Desondanks is het exacte mechanisme dat aan de basis ligt van de regulatie van de MDR1 expressie tot op heden nog niet volledig opgehelderd (Bates, 1999).

Een snelle inductie van MDR1 expressie werd vastgesteld in diverse cellijnen na contact met cytostatica. Chaudhary en Roninson (1993) onderzochten zowel het MDR1 mRNA via PCR als de flowcytometrische expressie en functie van P-gp in cellijnen afkomstig van chemotherapeutisch behandelde leukemieën of vaste tumoren. Sommige tumoren waren in vivo behandeld met cytostatica die geen P-gp substraten zijn. In de meeste cellijnen vonden de auteurs subpopulaties cellen terug met microscopisch zichtbare schade en met P-gp en MDR1 mRNA expressie. De cellijnen werden in vitro gekweekt zonder cytostatica in het kweekmedium en bij de leukemische cellijn K562 bleef de MDR1 expressie gedurende verschillende weken behouden.

Ook na blootstelling van cellijnen aan bepaalde stresserende omgevingsfactoren zoals “heat shock”, UV-bestraling en bepaalde carcinogenen stelde men een verhoogde expressie van het *MDR1* gen vast. Verhoogde MDR1 mRNA waarden werden eveneens teruggevonden na behandeling van neuroblastoomcellijnen en coloncarcinoomcellen met stoffen die een differentiatie uitlokken, zoals retinoïnezuur, natriumbutyraat, dimethylsulfoxide en dimethylformamide (Cornwell, 1996). Men stelde bij deze experimenten ook vast dat een toename in MDR1 mRNA niet altijd geassocieerd is met een verhoogde eiwitexpressie (Bates et al., 1989; Mickley et al., 1989).

Soms is er een niet-reversibele MDR1 overexpressie aanwezig, onder andere na genherschikkingen waardoor een “nieuwe” promotor permanent actief wordt. In recente experimenten onderzocht men cellen afkomstig van cellijnen en diagnostische leukemiestalen met een constitutioneel lage of afwezige P-gp expressie na blootstelling aan chemotherapie. Men kon in deze cellen herschikkingen van chromosoom 7q aantonen en een overexpressie van MDR1, wat erop zou kunnen wijzen dat genherschikkingen het *MDR1* gen kunnen activeren (Mickley et al., 1997). Pas na blootstelling van de cellijnen

aan hogere concentraties chemotherapie trad naast deze genherschikkingen ook een *MDR1* genamplificatie op (Knutsen et al., 1998).

Een snelle inductie van *MDR1* genexpressie, nog voor enige genherschikking mogelijk is, werd recent ook aangetoond na in vivo behandeling van sarcoommetastasen met doxorubicine. De *MDR1* genactivatie werd zowel gezien in de metastasen van vooraf behandelde patiënten, als bij nieuw-gediagnosticeerde patiënten (Abolhada et al., 1999). Men vermoedt dat de kans op een herschikking van het *MDR1* gen toeneemt tijdens deze acute inductie. Eenmaal er een herschikking gevormd is, blijft de promotor actief en is de MDR1 overexpressie irreversibel (Bates, 1999).

In andere cellijnexperimenten werd een sterke P-gp eiwitexpressie teruggevonden, met quasi geen verhoging van het MDR1 mRNA, wat suggereert dat de regulatie ook op translationeel of posttranslationeel niveau kan gebeuren (McClean and Hill, 1993).

2 Acute lymfoblasten leukemie (ALL) op kinderleeftijd

2.1 Prognostische kenmerken van ALL op kinderleeftijd

Ongeveer 80 % van de leukemieën op kinderleeftijd zijn acute lymfoblasten leukemieën. Met de huidige behandelingsschema’s, waarin rekening gehouden wordt met een aantal klinische en biologische risicofactoren, bereiken meer dan 95 % van de kinderen een complete remissie (CR) en bedraagt de lange-termijn EFS ongeveer 75 % (Riehm et al., 1990; Pui et al., 1998).

De belangrijkste risicofactoren bij ALL op kinderleeftijd zijn de volgende: leeftijd van de patiënt (kinderen jonger dan één jaar en ouder dan 10 jaar hebben een slechtere prognose); de leucocytose bij diagnose (meer dan 50 000 WBC per µl is ongunstig); het antwoord op de behandeling (meer dan 1000 leukemische cellen per µl in het bloed na één week behandeling met corticoïden is ongunstig); immuunfenotype van de blasten (waarbij de “voorloper” B-cel leukemieën de beste prognose hebben); DNA-indexen beneden 1.16 of groter dan 1.6; ongunstige cytogenetische kenmerken zoals de translocaties t(9;22) en t(4;11), de MLL genherschikking (11q23) en een bijna-haploïd chromosomenaantal; en de aanwezigheid van minimale ziekterest 30 dagen na start van de behandeling (MRD) (Pui et al., 1998; Gustafsson et al., 1998; Cavé et al., 1998; van Dongen et al., 1998). Ongeveer 25 % van de kinderen met ALL zullen tijdens of na de chemotherapeutische behandeling een recidief vertonen.

Het resistentiepatroon van leukemische blasten aan klinisch relevante cytostatica wordt in Nederland systematisch in vitro nagekeken. Een sterke in vitro resistentie correleert volgens deze onderzoekers met een slechte prognose bij kinderen met ALL (Pieters et al., 1991; Kaspers et al., 1997). Bijgevolg wordt een stratificatie van ALL patiëntjes op basis van deze “resistentie-profielen” aanbevolen (Pieters et al., 1998). Het meest onderzochte resistentiemechanisme bij ALL op kinderleeftijd is het transporteiwit P-gp (overzicht door Marie et al., 1999; Van den Heuvel-Eibrink et al., 2000;
Thesis Catharina Dhooge “Clinical significance of the Multidrug Resistance Protein (P-glycoprotein) in childhood cancer”, 2000).

P-gp wordt meestal bij minder dan 30 % van de kinderen met ALL teruggevonden bij initiële diagnose. In enkele studies worden meer P-gp positieve patiënten gezien bij recidief (Gekeler et al., 1992; Tafuri et al., 1995; Ivy et al., 1996; Dhooge et al., 1999). Andere auteurs zien geen verschil in P-gp positiviteit tussen diagnose en recidief (Pieters et al., 1992; Den Boer et al., 1998; Kanerva et al., 1998 en 2001). Sequentiële bepalingen van P-gp expressie bij dezelfde patiënten zowel bij diagnose als bij recidief, werd enkel in één prospectieve studie met lange follow-up beschreven (Dhooge et al., 1999). Van de 18 beschreven kinderen bleven er 11/13 negatief bij recidief. Twee van de vijf kinderen die positief waren bij diagnose scoorden negatief bij recidief, wat pleit tegen inductie van P-gp na vroegere chemotherapeutische behandeling.

In een aantal studies werd de correlatie tussen P-gp expressie en de klinische “outcome” van de patiënten bestudeerd. Kanerva et al. (1998) zag geen verschil in het bereiken van CR tussen P-gp positieve en negatieve patiënten. Marie et al. (1999) verklaart dit door de belangrijke rol van corticoïden, die geen P-gp substraat zijn, in het bereiken van remissie bij kinderen met ALL. In een latere studie (Kanerva et al., 2001) beschreven dezelfde auteurs géén verschil in EFS en OS na een follow-up van 4 jaar. Ook Wuchter et al. (2000) en Den Boer et al. (1998) vonden geen correlatie terug tussen P-gp expressie en functie bij initiële diagnose en antwoord op inductietherapie, recidieffrequentie en OS. Tafuri et al. (1995) bestudeerde een kleine groep van 33 ALL patiënten en vond wel minder CR terug bij patiënten met een positieve functionele test. Goasguen et al. (1993) en Dhooge et al. (1999) voerden prospectieve studies uit bij respectievelijk 23 en 102 kinderen met ALL en beiden vonden een significant minder goede EFS bij P-gp positieve kinderen. In de laatste studie werd een multivariant analyse van P-gp en andere prognostische parameters zoals leucocytose bij diagnose, aantal blasten in het perifeer bloed na één week corticotherapie, leeftijd bij diagnose, immuunfenotype, cytogenetische afwijkingen en DNA index uitgevoerd. Enkel P-gp expressie, leeftijd en leucocytose bij diagnose waren onafhankelijke voorspellende parameters voor een verhoogde kans op recidief.

Een aantal onderzoekers onderzochten MRP1 en LRP expressie op kinderleeftijd (overzicht door Van den Heuvel-Eibrinck et al., 2000). Men kan geen verband terugvinden tussen MRP1 expressie, in vitro resistentie aan daunorubicine of verlaagde retentie van daunorubicine in de blasten. Recidief ALL patiënten hebben geen duidelijk verhoogde MRP1 expressie in vergelijking met de novo patiënten (Den Boer et al., 1996 en 1998). Gurbuxani et al. (1998) bestudeerde 32 volwassenen en kinderen met de novo ALL en vond geen verband terug tussen MRP1 expressie en een invloed op CR of lange-termijn overleving. Den Boer et al. (1998) vond een significant hogere LRP expressie terug bij ALL patiëntjes die reeds meer dan één recidief doormaakten. Tussen LRP expressie bij de novo ALL en bij een eerste recidief was er echter geen duidelijk verschil. Ook werd geen verband gezien tussen LRP expressie bij diagnose en CR of OS. Dit werd bevestigd door Goasguen et al. (1996). Volm et al. (1997) daarentegen vond wel een significant verband terug tussen LRP expressie en lange-termijn overleving bij 38 kinderen met de novo ALL.

Ook de expressie van topoisomerase II werd onderzocht in ALL bij kinderen en bleek niet in verband te staan met klinische resistentie (Klumper et al., 1995). De verschillende genotypes van GST werden recent nagekeken bij 710 kinderen met ALL, behandeld volgens protocols van de Children’s Cancer Group. Men vond geen verband tussen de expressie van GST en de “outcome” van de patiënten (Davies et al., 2002).

Klinische studies over de nieuwere resistentie-eiwitten zoals BCRP zijn nog niet beschikbaar bij kinderen met ALL.

Eigen studies waarin het prognostisch belang van P-gp nagekeken wordt bij kinderen met ALL, zijn terug te vinden in hoofdstuk 3.

2.2 P-gp modulatoren en klinische "trials" bij leukemieën

Alle pogingen om MDR te omzeilen, hebben zich tot dusver geconcentreerd op de modulatie van P-gp. P-gp modulatoren zijn produkten die door directe interactie (bv. PSC 833 (Smith et al., 1998)) of door een competitieve inhibitie (bv. verapamil en ciclosporine A (Eytan et al., 1996)) de efflux van substraten van P-gp teniet doen.
Niet enkel resistentie tegen cytostatica maar ook resistentie tegen andere substraten van P-gp die gebruikt worden in de behandeling van tumoren, wordt op die manier aangepakt. Een voorbeeld van een dergelijk substraat is het MoAb anti-CD33 (gemtuzumab ozogamicin (CMA-676)) dat momenteel toegepast wordt als behandeling van volwassenen met CD33-positieve AML (Matsui et al., 2002).

De werking en enkele bijwerkingen van P-gp inhibitoren worden beschreven in deel 1.2 (De Moerloose et al., 1999).

De beste kandidaten voor "trials" in verband met MDR modulatie zijn de tumoren waarin de meerderheid van de cellen geen P-gp expressie hebben bij diagnose maar positief worden na chemotherapie. Voorbeelden hiervan zijn de leukemieën, lymfomen, multipel myelomen, neuroblastomen, sarcomen en borstcarcinomen. De criteria waaraan patiënten (in theorie) moeten voldoen om aan een "trial" deel te nemen zijn de volgende: de tumor moet refractair zijn aan chemotherapie; de tumor moet P-gp expressie vertonen en er moet een plasmaconcentratie voor de inhibitor bereikt (en volgehouden) kunnen worden zonder belangrijke toxiciteit te veroorzaken (Giaccone en Pinedo, 1996).

Studies met eerste generatie P-gp inhibitoren, zoals ondermeer verapamil en quinidine gaven geen overtuigend bewijs van een verbeterd antwoord op chemotherapie. Bovendien werden veel bijwerkingen van de modulatoren vastgesteld. In één van de eerste "trials" waarin hoge dosissen verapamil gegeven werden aan patiënten met een refractair ovariumcarcinoom, werd een ernstige cardiale toxiciteit gezien, ondanks relatief lage in vivo plasmaconcentraties van verapamil (Ozols et al., 1987).

De tweede generatie modulatoren (het niet-immuunsuppressief ciclosporine D analoog PSC 833 (Valspodar), VX 710 (Biricodar), GF120918 en het carboxamide derivaat GG918) werden specifiek ontwikkeld om P-gp te inhiberen en zijn in vitro ongeveer 10 keer meer potent dan verapamil (Boote et al., 1996; Sikic et al., 1997; Yanagisawa et al., 1999).
Praktisch gezien kunnen momenteel enkel ciclosporine A en PSC 833 in een voldoende hoge dosis toegediend worden om effectieve serumspiegels te bereiken zonder teveel toxiciteit te veroorzaken.
Omdat P-gp modulatoren ook de excretie en eliminatie van cytostatica verminderen door inhibitie van P-gp in de galcanaliculi en de renale tubuli, wordt in klinische "trials" de toegediende dosis cytostatica met ongeveer 25 tot 50 % gereduceerd (Sonneveld, 2000).

In de voorbije jaren werden een aantal fase II en fase III studies uitgevoerd bij volwassenen met AML (overzicht door Sonneveld, 2000).
In een SWOG (Southwest Oncology Group) fase III studie voegde men op een gerandomiseerde wijze al dan niet ciclosporine A toe aan de behandeling (met daunorubicine en cytarabine) van 226 hoog-risico AML patiënten. Er werd geen verschil gezien in de mortaliteit tijdens de inductie en in het bereiken van CR. Daarentegen waren de "relapse-free survival" en de overleving na 2 jaar beter voor de patiënten die ciclosporine A gekregen hadden (respectievelijk 34 % versus 9 %, P=0.031; en 22 % versus 12 %, P=0.046). Het effect van ciclosporine A op de overleving was het grootst bij patiënten met P-gp expressie. De belangrijkste bijwerking van ciclosporine A in deze studie was een geconjugeerde hyperbilirubinemie. De auteurs van deze studie schrijven het gunstig effect van ciclosporine A ondermeer toe aan het feit dat deze modulator ook in beperkte mate de retentie van daunorubicine in tumorcellen met MRP1 of BCRP expressie doet toenemen (List et al., 2001).

In 1995 startte de Eastern Cooperative Oncology Group (ECOG) een multicenter fase II studie waarin 37 hoog-risico AML patiënten met PSC 833 en mitoxantrone, etoposide en cytarabine behandeld werden. De toegediende behandeling werd goed verdragen. De bijwerkingen van PSC 833 (hyperbilirubinemie, veranderd bewustzijn en perifere neuropathie) waren transiënt en er werd geen ataxie, wat ook een mogelijke bijwerking van PSC 833 is, vastgesteld. Er was een antileukemisch effect in deze studie: twaalf patiënten (32 %) bereikten CR en vier een partiële remissie (Advani et al., 1999).

Ook resultaten van verschillende fase III trials met PSC 833 zijn momenteel bekend. De inclusie van patiënten in de studies van de ECOG en van de Cancer and Leukemia Group-B (CALGB) werd vroegtijdig stopgezet omwille van toxiciteit in de groep patiënten die PSC 833 kregen.
De ECOG studie vergeleek de behandeling met mitoxantrone, etoposide en cytarabine, al dan niet gecombineerd met PSC 833, bij (volwassen) patiënten met refractaire ziekte of recidief van AML. Men zag geen verschil in het bereiken van CR tussen de twee groepen. De patiënten worden nog verder opgevolgd om de EFS na te gaan (Greenberg et al., 1999).
De CALGB bestudeerde onbehandelde AML patiënten van minstens 60 jaar oud. De patiënten werden behandeld met cytarabine, daunorubicine en etoposide al dan niet in combinatie met PSC 833. Er werd een hogere mortaliteit kort na het starten van de behandeling vastgesteld bij de patiënten die PSC 833 toegediend kregen. In deze patiëntengroep werd ook minder CR bereikt. De EFS en OS na één jaar was vergelijkbaar tussen de groepen. Wel was de overleving van patiënten met functioneel P-gp die behandeld werden met PSC 833 beter dan van deze die geen PSC 833 toegediend kregen (mediane EFS respectievelijk 14 maand en 5 maand, p=0.07) (Baer et al., 2002).

In een Europese "trial" waarin PSC 833 samen met daunorubicine en cytarabine werd toegediend aan patiënten van minstens 60 jaar met een de novo AML, werd geen verschil gezien tussen de beide armen. Binnenkort worden deze resultaten geherevalueerd om na te gaan of een langere follow-up duur een verschil kan aantonen tussen de patiëntengroepen (Pieter Sonneveld, persoonlijke mededeling).

Sinds 1994 is een fase III studie bezig waarin kinderen met AML gerandomiseerd worden en al dan niet ciclosporine krijgen bij hun behandeling met mitoxantrone/etoposide tijdens

de consolidatiefase (Pediatric Oncology Group AML 9421 trial). Resultaten van deze studie zijn nog niet voorhanden.

Bij volwassenen met ALL en AML gebeurden een aantal studies waarin de co-expressie van meerdere resistentie-eiwitten nagekeken werd, bv. de expressie van P-gp en LRP of van P-gp en MRP1 samen. Uit deze studies bleek dat patiënten met een co-expressie minder goed scoorden qua CR, EFS en OS (overzicht door van den Heuvel-Eibrinck et al., 2000 en Sonneveld, 2000). Ook werd in vitro vastgesteld dat er tijdens de behandeling van P-gp positieve tumorcellen met de P-gp modulatoren ciclosporine A en PSC 833 een selectie van tumorcellen met LRP expressie optrad (List et al., 1998). Daarom is het zinvol om in de toekomst vooral modulatoren die meer dan één ABC-eiwit kunnen inhiberen of combinaties van modulatoren in klinische “trials” uit te testen. Voorbeelden van dergelijke modulatoren zijn GG120918 dat P-gp en BCRP inhibeert en VX 710 dat P-gp en MRP1 inhibeert (Marie et al., 2001).

Aangezien MDR1 expressie zeer acuut geïnduceerd kan worden (Abolhoda et al., 1998) suggereert Bates (1999) dat modulatoren beter ingeschakeld worden vooraleer een inductie kan ontstaan. Met andere woorden, in plaats van resistente tumoren te behandelen met een combinatie van chemotherapie en een modulator, zou het ontstaan van resistentie geanticipeerd moeten worden.
Dit werd ook gesuggereerd door Beketic-Oreskovic et al. (1995) die MES-SA sarcoomcellen behandelden met 40 nM doxorubicine. Zij stelden een selectie van resistente mutanten vast aan een incidentie van 1.8×10^{-6} per celgeneratie. Wanneer de cellen echter gelijktijdig met PSC 833 in contact gebracht werden, observeerde men dat de snelheid waarmee resistente klonen ontstonden 10 keer lager was.

3 Het neuroblastoom

3.1 Inleiding

De neuroblastomen maken 8 tot 10 % uit van alle tumoren op kinderleeftijd. De prognose van de kinderen ouder dan één jaar met een gemetastaseerd neuroblastoom (d.i. ongeveer 40 % van alle neuroblastoompatiënten) is slecht en de 3-jaar EFS bedraagt maximaal 30 % (Matthay et al., 1999; Berthold et al., 2000). Het neuroblastoom is daarom een typevoorbeeld van een tumor waarvoor het identificeren van parameters gecorreleerd met slechte prognose van belang is. Omdat het neuroblastoom een uitgesproken chemotherapieresistente tumor is en er bovendien therapeutische modulatoren bestaan voor P-gp, is het aangewezen de potentiële prognostische rol van P-gp na te kijken bij neuroblastomen.

ACTUELE INZICHTEN IN DE PROGNOSTISCHE KARAKTERISTIEKEN VAN HET NEUROBLASTOOM

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Tijdschrift voor Geneeskunde*, in press*

Actuele inzichten in de prognostische karakteristieken van het neuroblastoom

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Samenvatting:

Het neuroblastoom, een embryonale tumor typisch voorkomend op kinderleeftijd, kent een intrigerend klinisch verloop. In het ene geval ziet men een spontane of door behandeling geïnduceerde regressie of maturatie van de tumor tot een benigne ganglioneuroom; in andere gevallen maken de patiënten een agressieve en fatale ziekte door. De prognose wordt ondermeer bepaald door de leeftijd van de patiënt bij diagnose en het ziektestadium. Verder zullen ook de moleculaire en cytogenetische karakteristieken van de tumor het klinisch gedrag beïnvloeden. Het neuroblastoom is daarom terecht een typevoorbeeld van een tumor waar patiënten op basis van klinische en vooral biologische karakteristieken in prognostische groepen ingedeeld kunnen worden. Deze groepen worden op een aangepaste manier behandeld.
In dit artikel wordt een overzicht van de prognostische implicaties van volgende parameters gegeven: leeftijd van de patiënt, stadium van de ziekte, tumormerkers (NSE (neuron-specifiek-enolase), LDH (lactaat-dehydrogenase) en ferritine), histopathologie, expressie van tyrosine kinase receptoren voor bepaalde neurotrofines zoals NGF (Nerve Growth Factor), amplificatie van het *MYCN* oncogen, deletie van de korte arm van chromosoom 1, hyperdiploïd karyotype, telomerase activiteit, CD44 expressie en de expressie van genen betrokken bij chemotherapieresistentie (P-glycoproteïne en MRP1). Tenslotte worden de verschillende behandelingswijzen even toegelicht.

Abstract:

Neuroblastoma is the most common extracranial solid tumour in children. Neuroblastomas are derived from primitive neural crest cells and demonstrate diverse clinical and biological characteristics and behaviour. Whereas a subset of neuroblastomas has the capacity for spontaneous or induced maturation and/or regression, some exhibit an extremely malignant behaviour.
The outcome of the patient is dependent on age and stage, levels of lactate dehydrogenase, ferritin and neurone-specific enolase, pathology, and several molecular and cytogenetic characteristics such as tumour ploidy, MYCN gene amplification, allelic loss of chromosome 1p, expression of nerve growth factor receptors, CD44 expression, telomerase activity and expression of certain drug resistance proteins. Because prognosis has been shown to depend on these characteristics, biopsy sampling is nowadays recommended to enable classification of the patients in different prognostic subsets and to guide treatment.
This article reviews prognostic implications of various clinical and biological features and gives a brief overview of current treatment strategies.

Inleiding:

Het neuroblastoom werd in 1864 voor het eerst beschreven door Virchow (1). Neuroblastomen ontstaan in sympathogonia of voorlopercellen van het sympathisch zenuwstelsel afkomstig van de neurale lijst. De primaire tumoren ontstaan meestal op plaatsen waar het sympathisch zenuwstelsel normaal aanwezig is, namelijk in de ganglia langsheen de wervelzuil en in het bijniermerg.
De meeste tumoren worden abdominaal teruggevonden (gemiddeld 35 % in de bijnier en 30 % elders in het abdomen). De overige zijn thoracaal (19 %), in het kleine bekken (2 %) of in de hals (1 %) gelocaliseerd. In 12 % van de gevallen is de tumor elders

gelocaliseerd. De exacte oorsprong van de primaire tumor kan in 1 op 100 patiënten niet achterhaald worden. Soms zijn meerdere primaire tumoren aanwezig bij diagnose (1,2).
De localisatie van neuroblastomen is echter leeftijdsafhankelijk. Zo worden primaire tumoren in de bijnier vaker gezien bij kinderen ouder dan één jaar (2).
Op basis van de uitgebreidheid van de ziekte worden de patiënten in stadia onderverdeeld. Voorheen werd voornamelijk de klinische classificatie volgens Evans gebruikt (3). Deze classificatie werd door een groep van internationale experten tot de INSS (International Neuroblastoma Staging System) classificatie aangepast (4) (Tabel 1).

Tabel 1: INSS classificatie systeem (4)

Stad.	Uitgebreidheid:
St. 1	*Gelocaliseerd, macroscopisch volledig verwijderd, lymfeklieren negatief*
St. 2A	*Unilateraal, onvolledig verwijderd, lymfeklieren negatief*
St. 2B	*Unilateraal, +/- volledig verwijderd, positieve lymfeklieren ipsilateraal doch negatief contralateraal*
St. 3	*Tumor over de middellijn, +/- met pos. lymfeklieren of unilaterale tumor met pos. lymfeklieren contralat. of middellijn tumor met bilateraal pos. lymfeklieren*
St. 4	*Tumor uitgezaaid naar lymfeklieren op afstand, bot, beenmerg, lever of andere organen (behalve als st. 4S)*
St. 4S	*Gelocaliseerde tumor zoals gedefinieerd onder stadium 1 of 2 met meta's beperkt tot lever, huid of beenmerg*

Epidemiologie:
Ongeveer 8 à 10 % van alle maligne kindertumoren zijn neuroblastomen (2). Hiermee is het, na hersentumoren, de meest frequent voorkomende niet-hematologische maligniteit op kinderleeftijd. De jaarlijkse incidentie bedraagt ongeveer 1/100 000 kinderen jonger dan 15 jaar (5). Voor België betekent dit 15 tot 20 nieuwe patiënten per jaar.
Negentig percent van de patiënten is jonger dan 10 jaar bij diagnose. De mediane leeftijd bij diagnose bedraagt ongeveer 22 maanden, maar is significant lager bij gelocaliseerde tumoren in vergelijking met de meer uitgebreide vormen. Bij volwassenen is de ziekte uitzonderlijk.
Neuroblastomen komen wereldwijd voor. Er zouden iets meer jongens aangetast zijn. De verhouding tussen jongens en meisjes bedraagt 1.2/1 (1,2).

Erfelijkheid:
Het voorkomen van neuroblastomen is meestal niet genetisch bepaald. Heel zeldzaam werden familiale gevallen beschreven (6). Bij sommige patiënten wordt meer dan één primaire tumor gevonden. Men vermoedt dat dan wel een erfelijke voorbeschikkende factor aanwezig is.

Klinische kenmerken:
De klinische symptomen bij presentatie zijn afhankelijk van de primaire tumor en van de aanwezigheid van metastasen.
De primaire tumor veroorzaakt klachten door zijn lokalisatie, lokale uitbreiding of secretie van catecholamines. Neuroblastomen in de hals zijn zichtbaar als locale zwellingen. Thoracale tumoren kunnen aanleiding geven tot hoesten en dyspnoe. Abdominale neuroblastomen geven opzetting van de buik, buikpijn en constipatie als voornaamste klachten. Bij doorgroei van de tumor via de intervertebrale foramina naar het ruggenmerg toe (zandlopertumor), zal de patiënt zich met verlammingsverschijnselen presenteren.
Een verhoogde catecholamineproductie leidt tot klachten zoals zweten, vermagering, irritabiliteit, tachycardie en verhoogde bloeddruk. In sommige gevallen worden ook paraneoplastische verschijnselen zoals secretoire diarree met hypokaliëmie door VIP (vasoactief intestinaal peptide) secretie (d.i. het Kerner Morrison syndroom) of het syndroom van Kinsbourne gezien. Dit laatste wordt gekenmerkt door opsoclonus, myoclonieën en ataxie. Het wordt vermoedelijk veroorzaakt door immunologische kruisreacties van antilichamen gevormd tegen de tumorale cellen die ook tegen (normale) cellen in het cerebellum en het cerebrum reageren (1,2).

Neuroblastomen kunnen lymfogeen of hematogeen metastaseren. Metastasen worden teruggevonden in lymfeklieren of in het beenmerg, de lever, het bot en de huid. In zeldzame gevallen worden ook metastasen in de longen en de hersenen aangetroffen.
Beenmerginvasie geeft aanleiding tot pijn, anemie, thrombopenie en neutropenie. Botmetastasen geven bot- en gewrichtspijn.

Botpijn is een belangrijke, soms unieke en vaak miskende klacht bij een patiëntje met neuroblastoom. Orbitale botmetastasen zijn kenmerkend voor het neuroblastoom. Klinisch zijn ze te herkennen als een “brilhematoom”.

Diagnostische onderzoeken:
Men verricht steeds aanvullend onderzoek om de locale uitgebreidheid van de primaire haard vast te stellen en om metastasen op te sporen.
De bepaling van tumorparameters (neuron-specifiek-enolase (NSE), lactaatdehydrogenase (LDH) en ferritine) in het serum is nuttig zowel bij de diagnose als tijdens de follow-up (2). Een verhoogde 24-uurs urinaire excretie van catecholaminemetabolieten is een belangrijke diagnostische parameter, maar is slechts bij 90 % van de neuroblastoompatiënten aanwezig. Een scan met het isotoop metaiodo-benzylguanidine (MIBG) dat specifiek opgenomen wordt door catecholamine-secreterende cellen, zal bijgevolg ook slechts in 90 % van de gevallen positief zijn. Ander beeldvormingsonderzoek laat toe de tumor en metastasen te beoordelen (echografie, röntgenbeelden, CT scan of MRI, botscan). Men preleveert steeds beenmergstalen, bij voorkeur beenmergbiopten op twee verschillende plaatsen.
Omdat de prognose van een neuroblastoompatiënt in belangrijke mate door de biologische kenmerken van de tumorcellen bepaald wordt, zal men steeds een biopsie van de tumor verrichten.
Men stelt de diagnose neuroblastoom wanneer lichtmicroscopisch neuroblastoomcellen teruggevonden worden in biopsiemateriaal of wanneer er in het beenmerg neuroblastoomcellen gezien worden én er terzelfdertijd ook verhoogde concentraties catecholamines of catecholaminemetabolieten aanwezig zijn in urine of serum (1,2).
Neuroblastomen kunnen spontaan of geïnduceerd door therapie, uitrijpen of regresseren. Dit proces is mede afhankelijk van de leeftijd waarop de tumor ontdekt wordt. Indien de tumor zowel mature als immature elementen bevat, spreekt men van een ganglioneuroblastoom. Een ganglioneuroom daarentegen bestaat enkel uit uitgerijpte goedaardige cellen. Nabehandeling is hierbij niet nodig. Het onderscheid tussen ganglioneuroblastoom en neuroblastoom is klinisch niet van belang. Beide vormen worden immers als maligne beschouwd.

Prognostische kenmerken:
De prognose van de individuele patiënt is in grote mate afhankelijk van de leeftijd van de patiënt, het stadium van de ziekte en de aan- of afwezigheid van bepaalde biologische kenmerken of moleculaire factoren (Tabel 2).

Tabel 2: Moleculaire karakteristieken van neuroblastomen met potentiële prognostische informatie

Kenmerk:	Prognostisch ongunstig:
MYCN	Amplificatie (7-9)
DNA ploïdie	Euploïd (10)
1p 36.2 → ter	Deletie (11)
17q	Oververtegenwoordiging (12)
MRP1	Overexpressie (13)
Telomerase	Sterke activiteit (14,15)
TRK-A	Geen expressie (16,17)
CD44	Geen expressie (18)

MRP1: multidrug resistance associated protein 1
TRK-A: tyrosine kinase receptor A

Algemeen wordt aanvaard dat een leeftijd ouder dan één jaar, een stadium 4 neuroblastoom en *MYCN* amplificatie de belangrijkste ongunstige factoren zijn (1,2). De prognostische impact en de statistische onafhankelijkheid van de andere prognostische kenmerken moet nog in grotere studies aangetoond of bevestigd worden (7).
Gebaseerd op verschillende prognostische kenmerken werd een indeling van de neuroblastoom patiënten in drie subgroepen met verschillende prognose voorgesteld (2) (Tabel 3).

Tabel 3: Biologische/klinische neuroblastoom subtypes (2):

Kenmerk:	Type 1	Type 2	Type 3
MYCN	normaal	normaal	geamplificeerd
DNA ploïdie	hyperdiploïd of bijna triploïd	bijna diploïd of bijna tetraploïd	bijna diploïd of bijna tetraploïd
1p LOH	< 5 %	25-50 %	80-90 %
14q LOH	< 5 %	25-50 %	< 5 %
TRK-A expressie	sterk	laag	laag of afwezig
TRK-B expressie	laag	laag of afwezig	sterk
TRK-C expressie	sterk	laag of afwezig	laag of afwezig
Leeftijd	meestal < 1 jaar	meestal > 1 jaar	meestal 1-5 jaar
Stadium	meestal 1,2,4S	meestal 3,4	meestal 3,4
3-jaars overleving	95%	25-50 %	< 5%

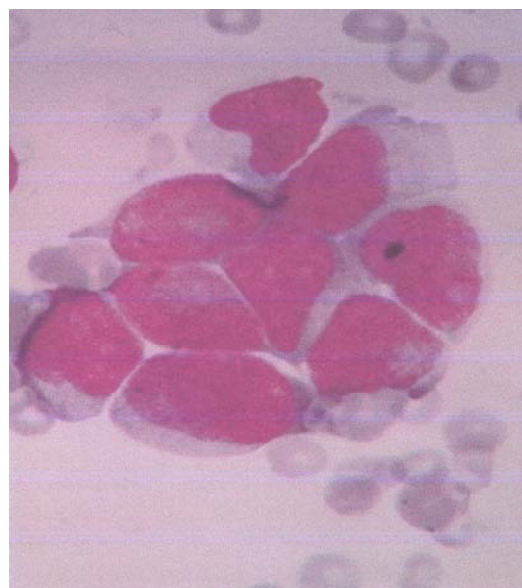
LOH: loss of heterozygocity

Leeftijd van de patiënt en stadium van de tumor:
Kinderen jonger dan één jaar hebben een veel betere prognose dan deze ouder dan één jaar, ongeacht het stadium van de tumor. Zo zal, in het geval van een gemetastaseerd neuroblastoom (stadium 4), de ziektevrije overleving na 5 jaar 60 % bedragen voor kinderen jonger dan één jaar en < 20 % voor oudere patiënten (19).
De prognose is gunstig voor patiënten met stadium 1 of 2 ziekte. De 5-jaars overleving bedraagt hierbij respectievelijk minstens 90 % en 85 % als er géén *MYCN* amplificatie aanwezig is (1). Patiënten ouder dan 1 jaar hebben dikwijls reeds gemetastaseerde ziekte bij diagnose (stadium 4) en overlevingskansen van slechts 10 tot 30 % (1,20-22). Bij *MYCN*-negatieve stadium 3 ziekte wordt vaak een grote inoperabele primaire tumormassa gevonden, maar is de prognose beter (65 tot 85 %) (1).
Bij kinderen jonger dan 1 jaar is er een speciale vorm van gemetastaseerde ziekte met een gunstige prognose, stadium 4S genoemd. De overlevingskans van deze kinderen bedraagt 85 % (1). Bij stadium 4S tumoren is een kleine tumor aanwezig met metastasen in lever en/of huid en/of beenmerg. Als er ook botmetastasen aanwezig zijn, gaat het echter niet om stadium 4S, maar wel om stadium 4. Bij stadium 4S patiënten kan spontane regressie van de primaire tumor en van de metastasen optreden. Een behandeling wordt enkel gegeven wanneer levensbedreigende situaties ontstaan door de snelle groei van de metastasen.

Tumormerkers: NSE, LDH, Ferritine
In verschillende studies werd aangetoond dat sterk verhoogde serumconcentraties van deze merkers met een slechte prognose gepaard gaan. Het prognostisch belang zou echter meer uitgesproken zijn bij gelocaliseerde tumoren dan bij stadium 4 tumoren (11,23-26).

Histopathologie, NGF (Nerve Growth Factor) en tyrosine kinase receptoren:
De drie klassieke histopathologische patronen (neuroblastoom, ganglio-neuroblastoom en ganglioneuroom) vertegenwoordigen een spectrum van maturatie en differentiatie.

Het typische neuroblastoom bestaat uit weinig gedifferentieerde kleine, uniforme cellen met dense hyperchromatische kernen en weinig cytoplasma. Soms worden neuropili terug-gevonden. Kenmerkend is de schikking in “rozetten”. (Figuur 1).



Figuur 1: Rozet-vormige schikking van neuro-blastoomcellen bij lichtmicroscopisch onderzoek.

Immuunhistochemische kleuringen van NSE, het ganglioside GD2 en neuroëctodermale antigenen kunnen de diagnose bevestigen.
Shimada gebruikt histologische kenmerken zoals de hoeveelheid Schwann cellen in het stroma en het aantal cellen in mitose of karyorrhexis, om neuroblastomen te classificeren (27). Aangezien bij deze histologische classificatie ook de leeftijd als parameter gebruikt wordt, is de Shimada classificatie bij vele anatomopathologen niet erg geliefd. Een internationale werkgroep heeft recent een universeel bruikbare histologische indeling opgesteld (28).
Sommige neuroblastomen kunnen spontaan regresseren en/of matureren tot een ganglioneuroom. Het benigne ganglioneuroom bestaat uit mature ganglioncellen, Schwann cellen en neuronen. De hypothese dat Schwann cellen, door de productie van NGF, een belangrijke rol in het maturatieproces spelen (29) wordt actueel echter betwist. NGF, behorend tot een familie neurotrofines waartoe ook BDNF (brain-derived neurotrophic factor), NT-3 (neurotrophin-3) en NT-4 (neurotrophin-4/5) behoren, heeft een antiproliferatief effect op neuroblastoom cellen en zorgt voor differentiatie.
De receptoren van deze neurotrofe substanties zijn tyrosine kinase receptoren (TRK receptoren). De belangrijkste ligand voor TRK-A, TRK-B en TRK-C is respectievelijk NGF, BDNF en NT3. De receptor van NT-4 is niet gekend, maar vermoedelijk interageert dit neurotrofine met TRK-B (2).
Een sterke TRK-A expressie zou met een uitstekende prognose correleren. Nakagawara et al. (16) toonden aan dat TRK-A expressie omgekeerd evenredig is met het voorkomen van *MYCN* amplificatie. Een tekort aan of een defect in de NGF receptor expressie kan tot het agressieve gedrag van sommige neuroblastomen bijdragen (17). De expressie van TRK-B is met *MYCN* amplificatie geassocieerd en gaat bijgevolg met een slechtere prognose gepaard. De expressie van TRK-C wordt bij voorkeur in lagere stadia en, net zoals TRK-A, niet in tumoren met *MYCN* amplificatie teruggevonden (2).

Genetische kenmerken:
Met behulp van karyotypering en moleculair genetisch onderzoek zoals FISH (Fluorescentie in Situ Hybridisatie) en LOH analyse (Loss of Heterozygosity) kan men in de maligne cellen van sommige neuroblastoompatiënten numerieke of structurele afwijkingen aantonen.
In neuroblastomen wordt een enorme variatie aan genetische afwijkingen teruggevonden (7). Meer dan 50 % van de neuroblastomen heeft een bijna normale DNA-inhoud (diploïd). Een triploïde DNA-inhoud wordt in ongeveer 30 % van de neuroblastomen teruggevonden. Tetraploïdie komt in 15 % van de gevallen voor. Triploïdie vindt men vaker terug bij lokale neuroblastomen (stadium 1 en 2) en bij jonge kinderen en is gecorreleerd met een betere prognose. Diploïde, pseudodiploïde en tetraploïde tumoren hebben een slechtere prognose (7). Hyperdiploïdie zou enkel bij zuigelingen gunstig zijn (2).
Karyotypering van neuroblastoomcellen levert soms aanwijzingen voor genamplificatie op. Extrachromosomaal chromatine (“double minutes”) of een intrachromosomale toename van genetisch materiaal (“Homogeneously Staining Regions”) correleert meestal met de amplificatie van het *MYCN* oncogen (30). *MYCN* is in normale omstandigheden gelocaliseerd op chromosoom 2p24. Het is een oncogen dat codeert voor een DNA-bindend eiwit (neuronale transcriptiefactor). Tot recent beschouwde men *MYCN* amplificatie als dé belangrijkste prognostische parameter in neuroblastomen (8). Deze amplificatie wordt bij 5 tot 10 % van de patiënten met lage ziektestadia en stadium 4S teruggevonden (1). Ongeveer 30 tot 40 % van de patiënten met stadium 3 of 4 vertoont *MYCN* amplificatie (1). Deze amplificatie is geassocieerd met snelle tumorprogressie, agressieve ziekte en slechte overleving. Daarnaast gaat de amplificatie vaak met een deletie op de korte arm van chromosoom 1 (1p) gepaard. Deleties op chromosoom 1p kunnen ook geïsoleerd voorkomen (11). Deze deletie is een frequent teruggevonden structurele afwijking in neuroblastoomcellen en zou eveneens met minder goede prognose gecorreleerd zijn (7,11). Andere deleties zijn ondermeer op de korte arm van het chromosoom 4 en op de lange arm van de chromosomen 11 en 14 terug te vinden. De deletie van consensusregio’s doet vermoeden dat er op die plaatsen tumorsuppressorgenen gelegen zijn.

De meest frequent aangetroffen genetische afwijking is een verhoogd aantal kopieën van de lange arm van chromosoom 17 (17q). Volgens de studie van Bown et al. zou dit de belangrijkste predictieve parameter voor slechte prognose (12).

Telomerase activiteit en CD44 expressie:
In 1994 werd door Kim et al. aangetoond dat er telomerase activiteit in bijna alle geïmmortaliseerde cellen en kankercellen aanwezig is (31). Dit wordt niet in normale cellen teruggevonden. Deze studies suggereren dat telomerase activiteit een verworven kenmerk is, geassocieerd met maligne transformatie. Hiyama

et al. bestudeerde de telomerase activiteit in neuroblastomen en kwam tot de conclusie dat er een zeer goede prognose gevonden wordt bij neuroblastomen met lage telomerase activiteit, en een zeer slechte prognose bij deze met zeer sterke activiteit (14). Dit werd bevestigd door Poremba et al. (15).

CD44 is een membranair glycoproteïne dat betrokken is bij interacties tussen cellen en tussen de cel en het interstitium. Combaret et al. onderzochten het belang van CD44 bij neuroblastomen en vonden in uni- en multivariaat analyse dat de afwezigheid van CD44 een belangrijke merker is van tumoragressiviteit. Zij bevelen het gebruik van CD44 als additionele biologische merker bij neuroblastomen aan (18).

Expressie van multidrugresistentie genen:
Neuroblastomen kunnen tijdens of na de behandeling een sterke resistentie aan chemotherapie vertonen. Deze resistentie neemt proportioneel toe met de intensiteit van de behandeling en zou het sterkst zijn bij recidief na beenmergtransplantatie (32).
Chemotherapie resistentie is een complex gebeuren dat door verschillende factoren, waaronder inefficiënte biodisponibiliteit van cytostatica, locale veranderingen in de tumor (zoals hypoxie), de expressie van multidrug-resistentie (MDR) genen en resistentie aan apoptose veroorzaakt kan worden (33).
Er zijn verschillende MDR genen beschreven. Het meest bekend zijn het *MDR1* genproduct/P-glycoproteïne (P-gp), de leden van de MRP (multidrug resistance associated protein)-familie (MRP1-7), LRP (Lung Cancer related Protein) en BCRP (Breast Cancer Resistance Protein) (34). P-gp is een membranaire pomp die verschillende cytostatica uit de cel pompt en hierdoor aanleiding geeft tot chemotherapie-resistentie. Deze pomp is uitvoerig bestudeerd in neuroblastomen, maar de prognostische betekenis blijft controversieel. Waar sommige auteurs P-gp expressie terugvonden in gemeta-staseerde en prognostisch ongunstige neuroblastomen (35-36), vonden anderen expressie in gelocaliseerde en beter gedifferentieerde tumoren (37-38). Ook in functionele studies waarin de pompactiviteit van P-gp bestudeerd werd, konden geen significante prognostische implicaties aangetoond worden (39).
Het MRP1 zou daarentegen wel in belangrijke mate met recidief en slechtere overleving geassocieerd zijn (13). Men vermoedt immers dat precies de resistentie van tumoren met *MYCN* amplificatie door MRP1 expressie veroorzaakt wordt (40).

Behandeling:
Neuroblastoom is een zeldzaam voorkomende ziekte. Toch bestond er tot voor kort weinig internationale samenwerking wat de behandeling betreft. Sommige landen hadden eigen protocollen. De verschillende Belgische centra sloten afzonderlijk aan bij een grotere groep, zodat een Belgisch kind met dezelfde ziekte toch vaak verschillend behandeld werd.
In 1994 startte een internationale Europese studie, LNESG (low stage neuroblastoma study group) genaamd. Het doel van deze studie was nagaan of heelkunde alleen als behandeling volstond bij patiënten met stadium 1 en 2 tumoren (*MYCN* amplificatie negatief). Als chemotherapie niet nodig bleek voor deze groep patiënten zouden de nadelige lange termijn effecten van de chemotherapie vermeden kunnen worden zonder de genezingskansen te compro-mitteren. Een andere doelstelling van de LNESG bestond in de studie van bijkomende prognostische factoren om die patiënten te identificeren waarvoor toch intensievere behandeling noodzakelijk was. Een groot aantal Europese landen werkte aan deze studie mee. Recent werd het beoogde aantal patiënten geïncludeerd en werd de studie afgesloten. De resultaten worden momenteel verwerkt en zullen binnenkort beschikbaar zijn.
Dankzij deze studie kwam er een vruchtbare samenwerking op gang, zodat besloten werd om ook voor de andere neuroblastoom stadia gemeenschappelijke protocollen op te stellen. Alleen door samenwerking kan men immers gerandomiseerde studies uitvoeren of het prognostisch belang van moleculair biologische factoren evalueren. Geen enkel land kan dit zelf realiseren binnen een redelijke termijn door het gebrek aan voldoende patiënten. Zo werd de SIOP (International Society of Pediatric Oncology)-Europe neuroblastoom groep opgericht.
Er werd een protocol opgesteld voor neuroblastoom patiënten jonger dan 12 maanden met neuroblastoom, INES genaamd (Infant Neuroblastoma Study). Hierin wordt de behandeling volgens de symptomen, het stadium van de ziekte en de moleculair biologische parameters aangepast waarbij speciaal rekening gehouden wordt met de jonge leeftijd van de patiënt.
Verder ging recent ook een protocol van start voor stadium 3 en niet heelkundig te verwijderen stadium 2 tumoren.
Voor patiënten met stadium 1 en heelkundig te verwijderen stadium 2 neuroblastomen is een versie van een nieuw protocol (LNESG II) klaar voor discussie. De prognose voor patiënten met stadium 4 neuroblastoom of een lager stadium

met *MYCN* amplificatie is slecht. De 5-jaar ziektevrije overlevingskansen bedragen tussen de 8 en 30 % (20,21). Volgens een grote Amerikaanse studie zijn de overlevingskansen voor deze groep patiënten iets beter wanneer ze naast een klassieke behandeling met chemotherapie, chirurgie en radiotherapie ook een autologe stamceltransplantatie en een onderhouds-behandeling met het vitamine A derivaat 13-cis retinoïnezuur krijgen (21). Deze resultaten zullen geverifieerd worden in een studie waarin enerzijds twee verschillende “conditioneringen” bij de autologe stamceltransplantatie toegepast worden en anderzijds een groep patiënten tijdens de onderhoudstherapie met retinoïnezuur behandeld wordt met immunotherapie (door toediening van chimere anti-GD2 antistoffen tegen neuroblastoom) in de hoop zo de genezingskansen nog te verbeteren. Ook voor patiënten met een slecht antwoord op de therapie of met recidief wordt een dergelijke behandeling voorgesteld. Dit laatste protocol wordt nu voorgelegd aan de verschillende ethische comités en zal binnenkort van start gaan.

In België werd in 1994 onder de auspiciën van de BSPHO (Belgian Society for Paediatric Haematology and Oncology) de BENG (Belgian Neuroblastoma Group) opgericht. In deze groep worden alle Belgische pediatrische oncologische centra vertegenwoordigd door een kinder-oncoloog. Ook andere specialisten uit diverse subdisciplines (radioloog, chirurg, cytogeneticus, patholoog, radiotherapeut, nucleaire genees-kundige, immunoloog, moleculair bioloog enz.) maken deel uit van deze groep. Dankzij deze samenwerking kunnen alle Belgische patiënten uniform behandeld worden en gebeurt zo nodig een centralisatie van technisch moeilijke of dure onderzoeken. Informatie betreffende de protocollen kan worden doorgegeven en beslissingen rond moeilijke patiënten kunnen samen genomen worden.

Besluit:

Het neuroblastoom is een klinisch en biologisch complexe tumor. Inzicht in de diverse moleculaire kenmerken van deze tumor zal in de toekomst leiden naar een nog verdere opsplitsing van patiënten in subgroepen met elk een verschillende prognose en therapiekeuze. De vermindering van chemotherapie bij laag risico patiënten zonder invloed op uiteindelijke overleving is één van de successen van deze aanpak.

Vooral bij de prognostisch meest ongunstige groep patiënten met overlevingskansen die de voorbije decaden slechts in beperkte mate verbeterden, zal doorgedreven onderzoek moeten leiden tot de identificatie van tumorspecifieke afwijkingen die als therapeutisch aangrijpingspunt kunnen dienen.

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3.2 Inductie van resistentie bij het neuroblastoom

In de kliniek wordt men vaak geconfronteerd met neuroblastoompatiënten die initieel goed reageren op de toegediende chemotherapie, maar die bij recidief sterk refractair zijn aan iedere behandeling (Matthay et al., 1999; Berthold en Hero, 2000).

Keshelava et al. (1998) bestudeerden neuroblastoomcellijnen afkomstig van patiënten die nog geen behandeling kregen, van patiënten met progressieve ziekte tijdens inductietherapie en van gerecidiveerde patiënten na intensieve chemo- en radiotherapie en beenmergtransplantatie. De in vitro cytotoxiciteit van verschillende concentraties melphalan, cisplatinum, carboplatinum, doxorubicine en etoposide werden bepaald. De IC_{90} waarden van de cytostatica waren het hoogst voor de cellijnen afkomstig van de patiënten die in vivo de zwaarste behandeling kregen en het laagst voor de cellijnen van de niet-behandelde patiënten.

Gelijkaardige observaties werden reeds vroeger beschreven (Kuroda et al., 1991). In die studie werd een verhoogde resistentie aan cisplatinum en doxorubicine vastgesteld in een neuroblastoomcellijn die verkregen werd na therapie, en dit in vergelijking met een cellijn afkomstig van dezelfde patiënt vooraleer enige behandeling gestart was.

Een bijkomende observatie in de studie van Keshelava was het feit dat de cellijnen hun resistentiepatroon behouden, zelfs na langdurig opkweken in medium zonder cytostatica. Dit contrasteert met andere studies waarin cellijnen opgekweekt werden in progressief hogere concentraties cytostatica om MDR1 expressie te induceren, en waarbij men na het verwijderen van het cytostaticum uit het kweekmedium een verdwijning zag van het MDR fenotype (Sugimoto et al., 1987).

3.3 Multidrugresistentie bij neuroblastomen: Literatuuroverzicht

3.3.1 P-glycoproteïne expressie in bijnierweefsel en neuroblastomen

In de jaren ‘80 werd de expressie van P-gp in normale weefsels en in vele tumoren van de mens onderzocht. Thiebaut et al. (1987) en Sugawara et al. (1988) vonden immuunhistochemische reactiviteit van het MoAb MRK16 in zowel de bijniercortex als het bijniermerg van volwassenen. Fojo et al. (1987) bevestigde de sterke expressie van MDR1 mRNA in het bijniermerg. Bijnieren van foetussen en pasgeborenen werden ook onderzocht en in deze weefsels werd géén P-gp expressie teruggevonden (Sugawara et al., 1988).

In 1989 publiceerden Goldstein en collega’s de resultaten van een grootschalige studie waarin MDR1 mRNA gemeten werd in meer dan 400 stalen, afkomstig van verschillende soorten tumoren. Hiervoor werden “bulk” tumorstalen geanalyseerd met een slot blot hybridisatie. Goldstein deelde de neuroblastomen in bij de groep tumoren die, onbehandeld, slechts sporadisch een hoge MDR1 mRNA expressie vertonen, maar die bij recidief na behandeling wel een duidelijk verhoogde MDR1 mRNA hebben.

In de daaropvolgende jaren werd het voorkomen van P-gp in neuroblastomen en het potentieel klinisch belang hiervan bestudeerd. Een overzicht van de gepubliceerde studies,

met vermelding van de gebruikte detectietechnieken, de onderzochte stalen, de resultaten en besluiten, zijn schematisch weergegeven in tabel 1.

Het gebruik van verschillende detectietechnieken maakt een goede vergelijking moeilijk. Een drietal tegenstrijdige stellingen kunnen onderscheiden worden: P-gp is een ongunstige parameter bij neuroblastomen; P-gp wordt eerder teruggevonden bij de prognostisch gunstige neuroblastomen (nl. de beter gedifferentieerde en de gelocaliseerde tumoren); en P-gp heeft prognostisch belang in bepaalde neuroblastoomsubtypes.

Tabel 1: Overzicht van publicaties over P-gp en MDR1 bij neuroblastomen

Referentie	Techniek	Stalen	Resultaat	Besluiten
Bourhis et al.,1989	northern en RNA slot blot	41 stalen (15 voor en 26 na chemo)	1/15 sterk pos bij diagnose; 11/26 sterk pos na chemotherapie	Toename van MDR1 expressie in behandelde NB; correlatie tussen overexpressie en slecht antwoord op chemo.
Goldstein et al.,1989	northern en slot blot	31 onbehandeld en 18 behandeld	55% van de onbehandelde pos; 83% van de behandelde pos; geen correlatie met andere prognostische parameters	Toename van MDR1 expressie in behandelde NB.
Goldstein et al.,1990	northern en slot blot		55% van de onbehandelde tumoren pos versus 70% van de behandelde	Toename van MDR1 expressie in behandelde NB. Uitselecteren van resistente tumoren na chemotherapie.
Bénard et al.,1990	northern blot	41 stalen	1/15 niet-behandelde NB pos versus 11/26 behandelde	Verhoogde P-gp expressie na chemotherapie.
Nakagawara et al.,1990	RNA slot blot	17 onbehandelde en 18 behandelde NB	16/17 onbehandelde patiënten pos, onafhankelijk van stadium	MDR1 expressie in gelocaliseerde en gedifferentieerde tumoren + omgekeerde correlatie met *MYCN* expressie.
Cordon-Cardo et al.,1990	IC met 3 MoAbs (o.a. C219)	182 tumoren, waaronder 7 NB	alle NB negatief	Geen positieve NB cellen.
Chan et al.,1991	IC, MoAbs: C219 en C494	67 tumorbiopten, onbehandeld	23 stad 1,2: neg 44 stad 3,4,4s: 31 neg en 13 pos	Alle neg patiënten in CR; slechts 6/13 pos patiënten in CR. Betere EFS en OS voor P-gp neg patiënten.
Bates et al.,1991	RNA in situ hybridisatie met RNAse protectie en IC (MRK16)	11 NB (7 voor en 4 na chemo)	Alle stalen MDR1 mRNA en 10/11 P-gp pos	Geen correlatie met leeftijd, stadium of overleving. Sterkere MDR1 expressie bij gedifferentieerde NB.
Corrias et al.,1991	northern blot	29 stalen, voor behandeling	5/29 pos, waarvan 3/8 stad 3 en 4	Geen correlatie van *MDR1* genamplificatie met prognose of EFS; *MYCN* en *MDR1* komen quasi niet samen voor.

Referentie	Techniek	Stalen	Resultaat	Besluiten
Favrot et al.,1991	immuunhistochemie, MoAb C219 (+ MRK16)	37 NB, waarvan 32 na chemo	geen positieve NB cellen	P-gp expressie beperkt tot niet-tumorale cellen.
O'Meara et al.,1992	immuunhistochemie, MoAb JSB1 (+ MRK16)	13 NB, waarvan 7 na chemo	geen positieve NB cellen	P-gp expressie beperkt tot niet-tumorale cellen.
Bénard et al.,1994	northern blot (en IC)	84 onbehandelde NB, alle stadia	20% pos dmv IC; 71% pos dmv northern blot (18/60 sterk en 42/60 intermediair)	Sterke MDR1 overexpressie significant gecorreleerd met slechte OS, ook na multivariant analyse. Northern blot overschat het aantal pos pat (door homogenisatie?)
Kim et al.,1995	immuunhistochemie, MoAb C219	30 NB (17 voor en 13 na chemo)	16/30 pos; meer pos stalen bij de histologisch beter gedifferentieerde NB	P-gp positiviteit is significant gecorreleerd met differentiatie.
Ramani and Dewchand,1995	NISH; IC met MoAb JSB1	30 stalen van 21 pat 9 tumoren ook na chemo getest	16/21 mRNA pos; 21/21 immuuncytochemisch pos	Geen correlatie met overleving (2 jaar); significante correlatie van P-gp met gunstige leeftijd en stad; NB na chemo meer gedifferentieerd dan voor chemo + alle P-gp pos.
Obana and Hashizume,1997	immuunhistochemie met MoAb MRK16	34 NB en 30 GNB (35 zuigelingen)	22/35 zuigelingen pos versus 9/20 oudere kinderen; 60% van de GNB pos	P-gp expressie in sympathische ganglioncellen weerspiegelt normaal maturatieproces; associatie met goede prognose.
Haber et al.,1997	RT-PCR	60 NB, 47 zonder *MYCN* amplificatie	hoge expressie wordt niet gezien bij *MYCN* amplificatie	MDR1 overexpressie is prognostisch belangrijk in de groep patiënten ouder dan één jaar en zonder *MYCN* amplificatie.
Dhooge et al.,1997	IC, MoAbs C219 en 4E3	23 pat; 25 stalen voor en 7 na chemo	10% van de stalen voor chemo pos	Enkel niet-gelocaliseerde tumoren P-gp pos; histologisch beter gedifferentieerde NB niet pos; geen ↑ expressie na chemo; geen correlatie met andere prognostische parameters.
Oda et al.,1997	RT-PCR; RNA dot blot; immuunhistochemie (Moabs JSB1 & C219)	13 stalen van 10 pat	11/13 pos in RT-PCR 6/13 pos in RNA dot blot 9/12 pos met immuunhistochemie	Geen correlatie tussen resultaten van immuunhistochemie en RT-PCR; geen significante correlatie met prognose.

Referentie	Techniek	Stalen	Resultaat	Besluiten
Kurowski and Berthold,1998	IC, MoAb C219	62 stalen, waarvan 2 na behandeling	14/62 positief	Geen correlatie met stad, histologie of *MYCN* expressie. EFS van stad 4 pat: iets minder goede overleving (P=0.069).
De Moerloose et al.,1999a	^{99m}Tc sestamibi beeldvorming	10 pat, allen voor chemo	1/10 ^{99m}Tc sestamibi positief	Goede concordantie tussen scintigrafische en flowcytometrische resultaten.
De Moerloose et al.,1999b	IC en flowcytometrie (MoAbs 4E3, MRK16); functionele test (flow)	26 stalen (NB + GN)	18/26 pos expressie 13/18 pos functie	Sterkste expressie in lage stadia en goed-gedifferentieerde tumoren. Sterkste activiteit in stad 4 NB.
Özcan et al.,1999	^{99m}Tc sestamibi beeldvorming	1 stad 4 pat	^{99m}Tc sestamibi negatief	^{99m}Tc sestamibi resultaat géén verband met klinische outcome; geen in vitro bepalingen van P-gp.
Burak et al.,1999	^{99m}Tc sestamibi beeldvorming	9 primaire haarden en 41 meta's van stad 4	0/9 primaire haarden pos; 16/41 meta's pos bij diagnose	^{99m}Tc sestamibi beeldvorming potentieel nuttige predictor van MDR in NBL; geen in vitro bepalingen van P-gp.
Bader et al.,1999	RT-PCR	40 NBL (20 voor en 20 na chemo)		Significant lagere MDR1 expressie bij tumoren met 1p-deletie ($p<0.05$); geen associatie met *MYCN* overexpressie.
De Moerloose et al., *submitted*	^{99m}Tc sestamibi beeldvorming; IC en flowcytometrie (MoAbs 4E3, MRK16); functionele test (flow)	14 NB, 1 GNB, 5 GN	16 tumoren ^{99m}Tc sestamibi neg, 2 tumoren ^{99m}Tc sestamibi pos; goede correlatie met in vitro testen	^{99m}Tc sestamibi beeldvorming is niet bruikbaar in detectie en staging. P-gp komt frequenter voor bij de lagere neuroblastoomstadia en de beter gedifferentieerde tumoren.

NISH: niet radioactieve in situ hybridisatie; IC: immuuncytochemie; pat: patiënt(en)

NB: neuroblastoom; GNB: ganglioneuroblastoom; GN ganglioneuroom

stad: stadium; pos: positief; neg: negatief

3.3.2 De prognostische waarde van P-gp in neuroblastomen

In de studie van Goldstein (1989) werd voor het eerst een toename in MDR1 RNA expressie gezien na behandeling van neuroblastomen met chemotherapie. Bovendien werd de verhoogde MDR1 expressie vooral in niet-gelocaliseerde stadia teruggevonden. In 1990 suggereerde Goldstein dan ook dat die significante toename na chemotherapie ontstaat door een uitselecteren van resistente cellen en bijgevolg gepaard gaat met een slechter antwoord op de toegediende chemotherapie. Bourhis et al. (1989) kwam tot gelijkaardige vaststellingen.

De belangrijkste studie waarin een duidelijk slechtere prognose voor P-gp positieve neuroblastomen gevonden werd, was deze van Chan et al. in 1991. Deze auteurs vonden enkel P-gp expressie terug bij de niet-gelocaliseerde (stadium 3 en 4) tumoren en besloten dat de P-gp status voor het starten van de behandeling duidelijk het succes van de therapie voorspelt bij de niet-gelocaliseerde neuroblastomen. Chan vond ook een correlatie terug tussen P-gp expressie en een meer ongedifferentieerd fenotype. Wanneer het eiwit niet gedetecteerd kon worden, was er een meer gedifferentieerde histologie.

In 1990 vond Bénard eveneens een verhoogde expressie van P-gp terug na behandeling met chemotherapie. In 1994 publiceerden dezelfde auteurs een studie waarin ze zowel immuuncytochemie als northern blot hybridisatie toepasten. In deze laatste studie stelden ze echter een belangrijke discrepantie vast in het aantal positieve patiënten, afhankelijk van de gebruikte techniek. Ze waarschuwden dan ook voor een mogelijke overschatting van positiviteit wanneer een detectietechniek gebruikt wordt die gehomogeniseerde stalen gebruikt.

In de studie van Kurowski en Berthold (1998) waar een immuuncytochemische “sandwich”techniek toegepast werd op tumorstalen, werd enkel bij de stadium 4 patiënten een tendens naar slechtere overleving gezien wanneer de tumoren P-gp positief waren (Logrank P= 0.069). Volgens deze auteurs kan P-gp expressie zeker niet het volledige beeld van chemotherapieresistentie in neuroblastomen verklaren.

Nakagawara et al. (1990) rapporteerde een MDR1 expressie in de lagere neuroblastoom stadia en in de tumoren met een duidelijke differentiatie bij histologisch nazicht. In diezelfde studie werd ook een omgekeerd evenredig verband vastgesteld tussen MDR1 en *MYCN* expressie. De hoogste *MYCN* expressieniveaus werden immers gevonden in de niet-gelocaliseerde neuroblastoom stadia en in de ongedifferentieerde tumoren. Kinderen met een sterke *MYCN* expressie hadden een slechte overleving; deze met een sterke MDR1 expressie hadden echter goede overlevingskansen.

Ook Corrias et al. (1991) en Bates et al. (1991) suggereerden dat een verhoogde MDR1 mRNA expressie frequent voorkomt in gelocaliseerde, beter gedifferentieerde en onbehandelde tumoren en tevens geassocieerd is met een goede prognose. Daarentegen zagen ze lagere MDR1 mRNA waarden in niet-gelocaliseerde, ongedifferentieerde en behandelde neuroblastomen.

Favrot et al. (1991) en O’Meara et al. (1992) pasten conventionele immuunhistochemische technieken toe en konden in geen enkele neuroblastoomcel P-gp terugvinden. Kurowski en

Berthold (1998) vonden wel P-gp expressie terug door middel van een gevoelige immuuncytochemische techniek, maar zagen geen significante associatie met histopathologische differentiatie van de tumor.

In 1995 bestudeerde Ramani 30 neuroblastoomstalen, waarvan er 9 getest waren voor en na chemotherapie. Ze paste een niet-radioactieve in situ hybridisatie toe om MDR1 mRNA op te sporen en eveneens een immuuncytochemische techniek. In haar studie bleek er een duidelijk verband te zijn tussen P-gp expressie en differentiatiestatus enerzijds en tussen P-gp en gunstige klinische parameters (leeftijd en stadium) anderzijds.

In een serie van 60 neuroblastomen konden Norris en collega's (1996) geen prognostisch belang van MDR1 expressie terugvinden. Wanneer daarentegen deze 60 patiënten onderverdeeld werden in subgroepen, kon wel een prognostische significantie teruggevonden worden. Een sterke MDR1 expressie was significant geassocieerd met een slechte outcome bij de 47 patiënten zonder *MYCN* amplificatie ($P<0.05$) en bij de 31 patiënten die bij diagnose ouder waren dan één jaar ($P<0.0001$) (Haber et al., 1997). Haber verklaart de tegenstrijdigheden rond prognostisch belang van P-gp bij neuroblastomen vooral door de diversiteit in de patiënten en tumoren (voorbehandeld of niet enz.) die in de verschillende studies geïncludeerd werden.

Recent verschenen studies waarin ^{99m}Tc sestamibi beeldvorming toegepast werd op neuroblastomen (Ozcan et al., 1999; Burak et al., 1999). Dit beeldvormingsonderzoek lijkt potentieel nuttig te zijn om in vivo P-gp gemedieerde MDR na te kijken. Hierop wordt nader ingegaan in hoofdstuk 4.

3.3.3 MDR1 inductie in neuroblastomen

Een verhoogde MDR1 expressie na blootstelling aan chemotherapie werd door meerdere auteurs beschreven (zie tabel 1: Goldstein et al., 1989; Bourrhis et al., 1989; Bénard et al., 1990). Ook in de studie van Ramani et al. (1995) werden 9 stalen onderzocht voor en na chemotherapie. Na chemotherapie waren deze tumoren histologisch beter gedifferentieerd en was er een sterkere MDR1 en P-gp expressie.
Enkel Kurowski en Berthold (1998) zagen bij hun twee patiënten die getest werden voor en na chemotherapie, géén verschil in P-gp expressie. Beide patiënten bleven immers negatief. In deze studie wordt echter niet vermeld of de histopathologie van de beide tumoren veranderde na de chemotherapie.

Het is bekend dat MDR1 expressie geactiveerd kan worden door bepaalde stimuli zoals chemotherapie of stoffen die differentiatie uitlokken. In 1989 zagen Bates en collega's een inductie van MDR1 expressie door de behandeling van neuroblastoomcellijnen met retinoïnezuur in vitro. Nochtans werd bij dit experiment niet het volledige MDR fenotype opgewekt: er was wel een duidelijk verhoogde P-gp expressie, maar de intracellulaire accumulatie van cytostatica veranderde niet.
Ook Ferrandis rapporteerde een toename van *MDR1* gentranscriptie in neuroblastoomcellen waarin neuronale differentiatie geïnduceerd werd door activatie van de proximale promotor (1993,1994).

Deze in vitro experimenten staven de bevindingen van ondermeer Nakagawara et al. (1990) die constateert dat MDR1 expressie voorkomt in goed-gedifferentieerde neuroblastomen en "down-gereguleerd" lijkt in slecht-gedifferentieerde. Volgens deze auteurs gedraagt MDR1 expressie zich als een merker van celdifferentiatie. Bijgevolg, is een verhoogde MDR1 of P-gp expressie na chemotherapie het gevolg van de selectie van resistente cellen of van een differentiatieproces dat door de chemotherapie geïnduceerd wordt?

Obana en Hashizume (1997) onderzochten bijnieren van volwassenen en van foetussen. Zij vonden géén P-gp expressie in het bijniermerg van de foetussen terug, terwijl dit wel het geval was bij volwassenen. Dit was ook vroeger vastgesteld door Sugawara et al. (1988). Terecht kan men zich daarom de vraag stellen of het verschijnen van P-gp expressie parallel verloopt met het normale maturatieproces. Dit wordt verder besproken in hoofdstuk 4.

3.3.4 MRP1 expressie in neuroblastomen:

MRP1 expressie werd door middel van PCR voor het eerst onderzocht in 5 neuroblastoomcellijnen en 25 tumoren door Bordow et al. (1994). Zij observeerden een correlatie van MRP1 expressie met de amplificatie en overexpressie van MYCN. Bovendien werd in cellijnexperimenten ook geobserveerd dat MYCN en MRP1 expressie daalden na behandeling met retinoïnezuur, terwijl de expressie van het *MDR1* gen toenam.

Norris et al. (1996) toonde aan dat een sterke expressie van het *MRP1* gen geassocieerd is met een toegenomen aantal recidieven en een kortere overleving ($P<0.001$). In die studie was er een 5-jaarsoverleving van 57 % voor patiënten met sterke MRP1 expressie, en een overleving van 94 % voor kinderen met lage MRP1 expressie. Bovendien was er een correlatie tussen de overexpressie van MRP1 en de overexpressie van MYCN. Ze suggereerden dat MYCN de expressie van MRP1 zou regelen en dat de slechte prognose van neuroblastomen met *MYCN* amplificatie te wijten zou zijn aan MRP1 overexpressie.

In 1997 toonde Norris aan dat een daling van de MRP1 expressie bekomen kon worden in vitro na MYCN inhibitie door gebruik van MYCN antisense RNA fragmentjes. Ook transfectie experimenten waarbij MYCN cDNA in neuroblastoomcellen ingebracht werd waarna de MRP1 expressie steeg, staafde deze hypothese (Haber et al., 1999).

Ook Bader et al. (1999) vond een significant hogere MRP1 genexpressie in tumoren met *MYCN* genamplificatie.
Andere onderzoekers konden daarentegen geen prognostische betekenis van MRP1 RNA expressie bij diagnose terugvinden (Matsunaga et al., 1998; Goto et al., 2000).

3.3.5 P-gp modulatoren bij vaste tumoren op kinderleeftijd

Dankzij de identificatie van P-gp als oorzaak van drugresistentie, leek men eindelijk in staat te zijn om ook refractaire tumoren te behandelen. Talrijke in vitro experimenten met cellijnen bewezen immers dat sterk resistente cellen opnieuw gevoelig konden worden na behandeling met P-gp inhibitoren. Dit was ook het geval voor neuroblastoomcellijnen (Cowie et al., 1998; Yanagisawa et al., 1999).

Er is een beperkt aantal studies over P-gp modulatoren bij tumoren op kinderleeftijd gepubliceerd.

In de studie van Cairo et al. (1989), waarin zowel hematologische maligniteiten (n=5) als vaste tumoren (één neuroblastoom stadium 4 en één hepatoblastoom) werden geïncludeerd, werd een continu infuus verapamil gecombineerd met etoposide en vinblastine. Respons werd verkregen bij 8/11 kuren zelfs met lage “steady-state” concentraties van verapamil. Bij deze studie kan men terecht opmerken dat de inclusie van hematologische aandoeningen, die gevoeliger zijn aan inhibitoren, het globale resultaat beïnvloedt. Bovendien waren alle patiënten vooraf met uiteenlopende therapieschema’s behandeld.

In 1991 werd een rapport gepubliceerd waarin men melding maakt van een partiële remissie door behandeling met verapamil en etoposide bij een kind van 9 maand oud met een neuroblastoom stadium 4 dat vooraf nauwelijks op de behandeling (vincristine, VP16, cyclofosfamide, carboplatinum/cisplatinum) gereageerd had (Phillips et al., 1991).
Dezelfde auteurs beschreven in 1993 hun resultaten van de combinatie verapamil met etoposide bij 9 kinderen met resistente tumoren (5 neuroblastomen, 2 gerecidiveerde neuroblastomen, 1 gerecidiveerd Ewing sarcoom en 1 gerecidiveerd Hodgkin lymfoom). Twee resistente neuroblastomen reageerden goed op de behandeling en bij 4 kinderen stabiliseerde de ziekte (3 neuroblastomen en 1 Ewing sarcoom) (Phillips et al., 1993).

In 1995 werden 20 patiënten met gerecidiveerde of resistente tumoren (1 Hodgkin lymfoom, 1 ALL, 1 Wilms tumor, 3 Ewing sarcomen, 1 osteosarcoom, 5 rhabdomyosarcomen en 8 neuroblastomen) behandeld met continu verapamil en etoposide. Er waren 6 partiële remissies na 2 kuren (bij 3 neuroblastomen en 3 rhabdomyosarcomen). Door variaties in het doseringsschema en de plasmaconcentratie van etoposide konden deze partiële remissies waarschijnlijk niet exclusief worden toegeschreven aan het effect van de inhibitor. Bovendien werd in deze studie ook geen P-gp status bepaald (Cowie et al., 1995).

Eénendertig kinderen met een P-gp positief rhabdomyosarcoom of ongedifferentieerd sarcoom (4 bij diagnose en 6 bij recidief), een P-gp positief neuroblastoom (4 bij recidief en 9 bij diagnose), of een klinisch resistent retinoblastoom (5 bij recidief en 3 bij diagnose) werden door Chan et al. (1992) behandeld met ciclosporine A en conventionele chemotherapie. Een positieve invloed op “response rate” en lange-termijn prognose werd vastgesteld. Dit bevestigde zich nadien voor retinoblastoompatiënten (Chan et al., 1996).

Schwartz et al. (2001) behandelden 22 osteosarcoom patiënten die resistent waren aan cisplatinum en doxorubicine met ciclosporine A. Vijftien tumoren werden onderzocht en 10/15 exprimeerden P-gp. Toch werd slecht weinig effect van ciclosporine A gezien: slechts 3 patiënten vertoonden een partieel antwoord. Bij de andere patiënten werd géén verandering of zelfs progressie gezien.

De modulator VX 710 kon in vitro de functie van P-gp en MRP1 inhiberen in zes neuroblastoomcellijnen die behandeld werden met verschillende cytostatica (Yanagisawa et al., 1999). In 1998 beschreef Rowinsky et al. de eerste resultaten van een fase I studie met VX 710. Klinische toepassingen van VX 710 bij tumoren op kinderleeftijd zijn niet

voorhanden. Recent werd ook het quinolone difloxacine nagekeken als modulator van MRP1. Neuroblastoomcellijnen met MRP1 expressie werden in contact gebracht met cytostatica (waaronder een aantal MRP1 substraten) en met difloxacine. Dit antibioticum kon in vitro de gevoeligheid voor de MRP1 substraten doen toenemen, en is dus ook een potentiële kandidaat om in klinische “trials” nagekeken te worden (Norris et al., 2001).

HOOFDSTUK 2

DETECTIE VAN P-GLYCOPROTEÏNE

P-gp kan op verschillende manieren gedetecteerd worden (Weinstein et al., 1991; van der Heyden et al., 1995). MDR1 mRNA kan bepaald worden via Northern blot of slot blot analyse, in situ hybridisatie of (RT-)PCR. De meest gevoelige van deze MDR1 RNA detectiemethodes is de (RT-)PCR. Aangezien deze technieken steeds gehomogeniseerd "bulk" tumormateriaal gebruiken, is er een grote kans op contaminatie. Bovendien is er geen strikte correlatie tussen het mRNA gehalte en het eiwitgehalte in de cel. In dit doctoraatsonderzoek verkozen we daarom om rechtstreeks het P-gp eiwit te detecteren met gevoelige immunologische technieken (een immuuncytochemische techniek en een flowcytometrische immunologische test) en om functioneel onderzoek te verrichten om een idee te krijgen over de activiteit van het gedetecteerde P-gp. Dit functioneel onderzoek gebeurde flowcytometrisch en door middel van een in vivo scintigrafisch onderzoek.

1 Controlecellijnen

De controlecellijnen die bij ieder in vitro experiment gebruikt werden, zijn de humane myeloïde leukemie cellijn K562 en diens multidrugresistente, P-gp exprimerende kloon K562/VLB (Van Acker et al., 1993). Beide cellijnen werden opgekweekt in minimal essential medium (MEM Gibco-BRL, Paisley, Schotland). Aan het medium van de cellijn K562/VLB werd 20 ng/ml vinblastine toegevoegd.

2 Monoklonale antilichamen

In overeenstemming met de consensus-aanbevelingen werden steeds twee verschillende monoklonale antilichamen (MoAbs) gebruikt (Beck et al., 1996; Marie et al., 1997). De optimale concentratie van de MoAbs die in onze studies gebruikt werden, werd in onze laboratoria zelf bepaald aan de hand van de controlecellijnen en klinische stalen.

Initieel werden de MoAbs C219 en 4E3 (Dako Corporation, Carpinteria, USA) gebruikt in de immuuncytochemische test in een concentratie van 20 µg/ml en 10 µg/ml voor respectievelijk C219 en 4E3. C219 herkent een intern epitoop van de beide P-gp isovormen MDR1 en MDR3 en is bijgevolg minder specifiek. Bovendien werden ook kruisreacties met myosine beschreven (Weinstein et al., 1991). Het MoAb 4E3 herkent een extern P-gp epitoop.

In flowcytometrische immunologische testen worden bij voorkeur MoAbs gebruikt die binden aan externe epitopen. Om MoAbs te kunnen toepassen die een intern epitoop herkennen, is er immers een permeabilisatieprocedure nodig die de "scatter"-eigenschappen van de bestudeerde cellen verandert en hierdoor de analyse moeilijker maakt.
Daarom werden, in de latere studies waarin gelijktijdig immuuncytochemie en flowcytometrie toegepast werd, twee externe MoAbs gebruikt, nl. 4E3 en MRK16 (Kamiya Biomedical Company, Thousand Oaks, USA). De optimale concentratie voor 4E3 in de flowcytometrische testen was 7.5 µg/ml. Voor MRK16 werd immuuncytochemisch en flowcytometrisch telkens 5 µg/ml gebruikt.

3 Immuuncytochemische APAAP techniek

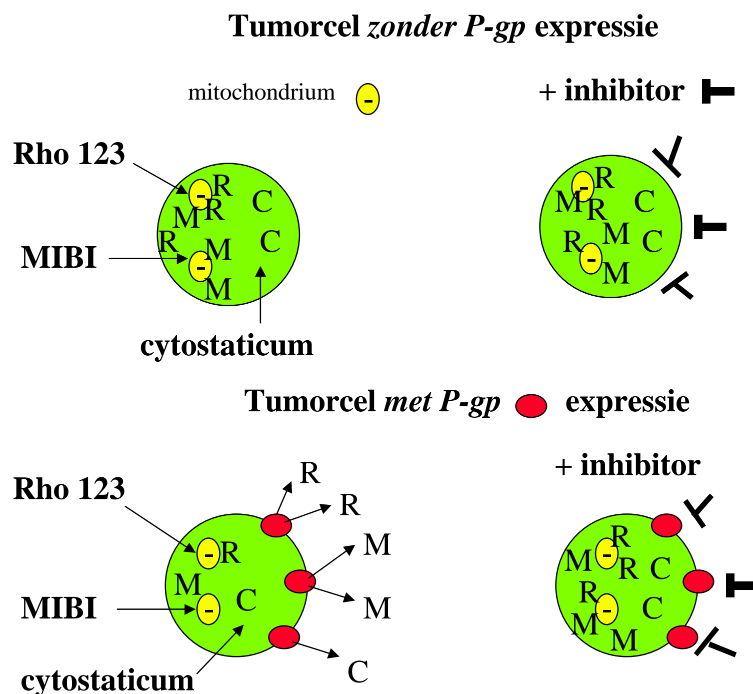
De immuuncytochemische techniek zoals door ons toegepast, werd voor het eerst beschreven in 1994 (Dhooge et al., 1994). Bloed- of beenmerguitstrijkjes en doppreparaten van vaste tumoren worden gedurende 24 uur gedroogd en nadien ingevroren (-20°C). Na fixatie in glutaaraldehyde 0.0125% in PBS (Phosphate Buffered Saline, pH 7.2) bij 4°C gedurende 30 seconden, worden de stalen gedurende 30 minuten bij kamertemperatuur geïncubeerd met het gebruikte MoAb of met een isotypische controle ($IgG2_a$). Nadien wordt de alkalische fosfatase-anti-alkalische fosfatase (APAAP, Dakopatts, Glostrupp, Denemarken) kleurreactie uitgevoerd, zoals beschreven door Cordell et al. (1984). De APAAP incubatiestappen worden een tweede keer herhaald om de gevoeligheid van de techniek te verhogen. Nadien worden de plaatjes ontwikkeld met Fast-Red TRsalt (Sigma, St. Louis, USA), tegengekleurd met gallocyanine en gemonteerd.
In overeenstemming met de consensus-aanbevelingen wordt geen "cut-off" waarde gebruikt en wordt het staal positief genoemd wanneer een rode aankleuring op de celmembraan van minstens één maligne cel gezien wordt.

4 Immunologische en functionele flowcytometrische testen

De flowcytometrische testen worden in detail beschreven in de volgende manuscripten (De Moerloose et al., 2000; Swerts et al., *submitted*) en in de hoofdstukken 3 en 4 (De Moerloose et al., 1999b; De Moerloose et al., *submitted*).

De immunologische test is een multiparameter techniek, m.a.w. de cellen worden niet enkel geïncubeerd met de MoAbs gericht tegen P-gp, maar gecombineerd met MoAbs om de bestudeerde celpopulatie te identificeren.

De functionele test is gebaseerd op de accumulatie en de retentie van het fluorescent P-gp substraat Rhodamine 123 (Rho 123). Deze lipofiele en kationische kleurstof diffundeert door de celmembraan en accumuleert in de mitochondria van de cel. Wanneer P-gp actief is, zal er minder intracellulair Rho 123 aanwezig zijn. Het verschil tussen P-gp negatieve en P-gp positieve cellen wordt in deze functionele test duidelijk door een gelijktijdige incubatie met de P-gp inhibitor verapamil. Enkel de cellen met een actieve P-gp pomp zullen in aanwezigheid van verapamil een hogere intracellulaire Rho 123 fluorescentie vertonen in vergelijking met de cellen die niet geïncubeerd worden met verapamil (Figuur 4).



Figuur 4: Schematische voorstelling van de opname en retentie van P-gp substraten in gevoelige en resistente cellen, al dan niet in aanwezigheid van een P-gp inhibitor (R: Rho 123; M: MIBI (^{99m}Tc sestamibi); C: cytostaticum).

P-GLYCOPROTEIN IN CHILDHOOD ACUTE LEUKAEMIA: COMPARISON OF THREE DIFFERENT DETECTION TECHNIQUES

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Analytical Cellular Pathology, *submitted*

P-glycoprotein in childhood acute leukaemia: comparison of three different detection techniques

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Abstract
The multidrug resistance protein, P-glycoprotein (P-gp), plays an important role in chemotherapy failure. Studies evaluating the clinical significance of P-gp in childhood acute leukaemia are hampered by a lack in standardised detection techniques, leading to divergent data. In this study, three different assays for P-gp detection in childhood acute leukaemia are compared. Samples from 110 children were analysed using an immunocytochemical test, a flow cytometric immunological test and a flow cytometric functional assay. P-gp expression was evaluated using two monoclonal antibodies (4E3 and MRK16) and P-gp activity was determined using rhodamine 123 in combination with the P-gp inhibitor verapamil. In this study, only the functional assay showed a significant difference in P-gp activity between ALL and ANLL patients. No increased P-gp expression or activity was found at relapse. Using the functional assay as a reference, sensitivity, specificity and predictive values of the immunological tests were calculated. The inter-assay agreement was poor between immunocytochemistry and flow cytometry. The agreement was fair between both flow cytometric tests. We conclude that all three techniques can be used to study P-gp expression and/or activity in childhood acute leukaemia but we recommend immunocytochemistry in combination with a functional flow cytometric assay.

Keywords: MDR, P-glycoprotein, childhood leukaemia, immunocytochemistry, flow cytometry

Introduction

Treatment results in childhood acute leukaemia have improved markedly during the past 20 years. However, over 50% of the children diagnosed with ANLL relapse. In addition, approximately 20% of the children with ALL relapse and can not be cured with modern therapy. The major cause of treatment failure in childhood acute leukaemia is the emergence of multidrug resistance. Several mechanisms of resistance are known but the best studied one is the overexpression of a 170 kD transmembrane protein, P-glycoprotein (P-gp). This ATP-dependent efflux pump extrudes a variety of lipophilic, cytotoxic compounds out of the cell and hence decreases their intracellular concentration and toxicity [10]. Different structurally and functionally unrelated chemotherapeutic agents, such as anthracyclines, vinca alkaloids and epipodophyllotoxins are substrates of the P-gp pump.

Despite profound research, the prognostic value of P-gp in paediatric acute leukaemia remains contradictory [6,8,12,13,14]. One of the reasons for the variability in the published data is the variety in applied detection techniques. This problem has been studied in a number of international workshops and several recommendations regarding sample preparation, use of adequate controls and interpretation and reporting of data have been formulated [2,3,9,17,18]. Generally, flow cytometry and immunocytochemistry are preferred to immunoblots, Northern blots and polymerase chain reaction (PCR)-based tests. Especially, the use of a functional flow cytometric assay has been promoted because it allows, in contrast with the immunological tests, the detection of functional P-gp pumps [3,17,18].

In the present study, three different assays for P-gp detection in childhood acute leukaemia are compared. Samples of both ALL and ANLL patients were examined by an immunocytochemical test, a flow cytometric immunological test and a flow cytometric functional assay.

Since the latter has been recommended as the most clinically relevant test, it was used as a reference method to calculate the sensitivity, specificity and predictive values of the immunological tests. Finally, the correlation between the different techniques was evaluated.

Materials and methods

Patient groups and sample preparation

From January 1995 to September 2001, 125 samples from 110 consecutive patients were evaluated for P-gp expression and/or function. In

76 children the diagnosis of ALL was made. In 17 patients ANLL was diagnosed. Samples from 25 ALL and 7 ANLL patients at relapse were also included in the study. In 9 ALL and 6 ANLL patients, P-gp expression and/or activity was evaluated both at diagnosis and at relapse. All three techniques were applied to 105 out of 125 samples (84 %).
Leukaemic cells were collected from bone marrow (BM) or peripheral blood (PB). For immunocytochemistry, BM and/or PB smears were air-dried for 24 hours at room temperature and stored unfixed at –20 °C until use. For flow cytometry, mononuclear cells from BM and/or PB were isolated using density gradient centrifugation on Fycoll-hypaque. The cells were stored at 4 °C until use and analysed within 24 hours after prelevation.

Control cell lines
Samples of the well-characterised human chronic myeloid leukaemia cell line K562 and its P-gp expressing resistant clone K562/VLB20 were included in each immunological and flow cytometric experiment as a negative and a positive control, respectively [21].

Monoclonal antibodies
In both immunological assays, P-gp was detected by two monoclonal antibodies (MoAbs) which recognise different extracellular P-gp epitopes, namely 4E3 (Dako Corporation, Carpinteria, USA) and MRK16 (Kamiya Biomedical Company, Thousand Oaks, USA). The optimal concentration for 4E3 was 10 µg/ml and 7.5 µg/ml in the immunocytochemical and the flow cytometric assay, respectively. For MRK16, a concentration of 5 µg/ml was used in both tests.

Immunocytochemistry
The expression of P-gp on leukaemic blasts was demonstrated by the alkaline phosphatase- anti-alkaline phosphatase (APAAP) immunocyto-chemical technique as described previously [7]. Briefly, the slides were fixed in 0.0125% glutaraldehyde for 30 seconds at 4 °C, washed and incubated with the MoAbs in a humidifier at room temperature for 30 minutes. The APAAP (APAAP-Dakopatts, Glostrupp, Denmark) staining procedure was performed as prescribed by the manufacturer [4]. A higher sensitivity was obtained by a repeated incubation with the rabbit anti-mouse antibodies and the APAAP complex for another 5 minutes. Subsequently, the slides were developed with Fast-Red TR Salt (Sigma, St. Louis, USA) and counterstained with gallocyanin. Isotypic matched controls and controls without the primary antibody were simultaneously evaluated to exclude atypical or background staining. The slides were evaluated by three independent observers. In agreement with the consensus recommendations, no arbitrary minimal cut-off point was used [2]. A mean of 300 blasts was examined per slide. In case of low cellularity, at least 50 cells were analysed. A patient was defined positive when at least one blast with a red staining pattern along the cell membrane was observed. In the majority of the samples, the three observers came to the same conclusion. In case of disagreement (10%), samples taken at the same time and stored at – 20 °C were reanalysed until a consensus was reached.

Flow cytometric detection of P-gp expression
Cells ($5x10^5$) were incubated for 30 minutes with the isotypic control antibody IgG2a (Dako Corporation, Glostrup, Denmark) or the P-gp specific MoAbs in parallel samples. Cells were washed twice and incubated for 20 minutes with fluorescein isothiocyanate- or phycoerythrin-labeled goat $F(ab')_2$ anti-mouse IgG (Caltag Laboratories, Burlingame, UK). Leukaemic blasts were identified by specific markers in a multicolour assay. The fluorescence was analysed on a FACSort flow cytometer (Becton Dickinson, Erembodegem, Belgium). At least 10.000 events were counted and analysed with the Cell Quest software (Becton Dickinson, Erembodegem, Belgium).
P-gp expression was scored quantitatively by measuring the mean fluorescence channel shift of the gated cell population, when comparing the relative fluorescence of the MoAb and the isotypic control antibody. In order to compare the results of this flow cytometric assay with those of the immunocytochemical test, a cut-off value was used. A sample was considered positive when the mean fluorescence of the cells stained with 4E3 or MRK16 was at least 10% higher than the mean fluorescence of the cells stained with IgG2a, resulting in a mean fluorescence index of at least 1.10, as previously described by De Moerloose [5].

Table 1: Expression and activity of P-gp

		Immunocytochemistry			Flow cytometry			Functional
	Total	4E3	MRK16	Both	4E3	MRK16	Both	
De novo ALL	76	19/75	27/76	16/75	16/60	11/57	5/57	15/75
		25%	36%	21%	27%	19%	9%	20%
Relapse ALL	25	6/25	10/24	5/24	3/22	1/19	0/19	6/25
		24%	42%	21%	14%	5%	0%	24%
De novo AML	17	7/17	5/16	5/16	8/17	5/14	4/14	7/17
		41%	31%	31%	47%	36%	29%	41%
Relapse AML	7	2/7	2/6	2/6	4/7	1/7	1/7	4/7
		29%	17%	17%	57%	14%	14%	57%

Flow cytometric detection of P-gp activity
Cells ($1x10^5$) were incubated for one hour at 37 °C with the P-gp substrate Rhodamine 123 (Rho123) (200 ng/ml) (Sigma, St. Louis, USA) in the presence or the absence of the P-gp modulator verapamil (10 µM) (Knoll, AG, Ludwigshafen, Germany). The intracellular Rho 123 accumulation was measured using FACS analysis. Cells were washed twice and incubated with or without verapamil for another hour at 37 °C. The remaining intracellular Rho123 concentration was measured with a flow cytometer after the efflux period. Results were expressed as a ratio: the mean intracellular Rho123 fluorescence in the presence of verapamil was divided by the mean intracellular Rho123 fluorescence in the absence of the inhibitor. A cut-off value was applied to compare the results with those of the immunocytochemical assay. Samples were considered positive when at least 10% of the cells exhibited a Rho123 efflux which could be inhibited by verapamil [5].

Statistical analyses
Differences in P-gp expression rates and activities were analysed using chi square tests. Inter-assay agreement was quantified using the Kappa (κ) statistic. A κ value of < 0.20, 0.21-0.40, 0.41-0.60, 0.61-0.80, 0.81-1.00 is interpreted as a poor, fair, moderate, good and very good strength of agreement, respectively [1].

Results

P-gp expression rates and P-gp activity in childhood ALL and ANLL
In this study, 125 samples from 110 consecutive patients were evaluated for P-gp expression and/or function. In 18 patients, the immunological flow cytometric assay could not be evaluated due to technical problems. From 2 patients, there was not enough material available. All three techniques were applied to 105 out of 125 samples (84 %). The results are summarized in Table 1.

The lymphoblasts of sixteen children (21%) with a de novo ALL scored positive for both MoAbs using the immunocytochemical APAAP technique. The immunological flow cytometric assay was positive in 27% (16/60) and 19% (11/57) for 4E3 and MRK16, respectively. Only 9% (5/57) of these patients was positive using both MoAbs. Fifteen children (20%) showed a Rho123 efflux which could be reversed in the presence of the P-gp inhibitor verapamil.
Five relapsed ALL patients (21%) were positive for both MoAbs using the APAAP assay. In 14% (3/22) of these children, a positive flow cytometric result was found using 4E3. Only one patient was positive for MRK16. Six relapsed patients (24%) showed a reversible Rho123 efflux.
In the group of de novo ANLL patients, 5 out of 16 patients (31%) scored positive for both MoAbs using the APAAP assay. The immunological flow cytometric assay using 4E3 and MRK16 was positive in 47% (8/17) and 36% (5/14) of these patients, respectively. Four patients (29%) were positive for both MoAbs. An enhanced efflux was seen in 7 de novo ANLL patients (41%).
We included 7 ANLL patients at relapse in this study. This group was too small for statistical analysis.
The concordance between the results of both immunological assays using 4E3 was very good (χ^2=4.56 p=0.035). In contrast, there was no significant association between the results of the immunological tests using MRK16 (χ^2=0.38 p=0.545).
The functional assay showed a statistically significant difference in P-gp activity between ALL and ANLL patients (χ^2=5.08 p=0.025). There was no significant difference between both groups using the immunological tests.
In this study, we found no statistically significant increase in P-gp expression or activity at relapse (χ^2=0.06 p=0.80; χ^2=0.82 p=0.37; χ^2=0.30 p=0.59

for APAAP, immunological flow cytometric assay and functional test, respectively)

Samples from 9 ALL and 6 ANLL patients were evaluated for P-gp expression and/or activity both at diagnosis and at relapse using a least one of the three detection techniques. The results are summarized in Table 2. At diagnosis, 4 ALL patients were negative for all tests. Of these patients, only one patient scored positive at relapse. Also one ANLL patient lacked functional P-gp pumps at diagnosis but scored positive at relapse. On the contrary, 2 ALL and 2 ANLL patients, positive for one of the assays at diagnosis, were negative for both tests at relapse.

Table 2: Results of patients tested at diagnosis and at relapse

ALL patients		Relapse	
		Positive	Negative
	Positive[1]	3	2
Diagnosis	Negative[2]	1	3

ANLL patients		Relapse	
		Positive	Negative
Diagnosis	Positive1	3	2
	Negative2	1	0

[1] Sample scored positive for the immunological or the functional flow cytometric tests
[2] Sample scored negative for both flow cytometric assays

Comparison of the three detection techniques

Table 3 summarizes the different test results obtained in 105 samples tested with all three methods. Concordant immunological test results were found in 62 patients (59%). In 46 children, both immunological assays were negative. Sixteen patients scored double positive. Of the 62 children with concordant immunological results, 45 (73%) showed a corresponding functional test result. In 38 patients, all tests were negative. Seven patients scored positive for the three assays. Eight patients (8%) were negative for the immunological tests but showed a reversible Rho123 efflux. Nine patients (9%) scored positive for the immunological assays but were negative for the functional test. Discordances between the immunological tests were seen in 43 patients (41%).

As stated in the introduction, the functional assay was used as a reference method to calculate the sensitivity, specificity and predictive values of the immunological tests.

The sensitivity of both immunological assays is low (41% for APAAP and 56% for the flow cytometric immunological assay). The specificity, positive predictive value and negative predictive value was 65%, 29% and 76% for APAAP and 72%, 41% and 82% for the flow cytometric immunological assay.

The inter-assay agreement was poor between APAAP and the flow cytometric immunological assay (κ=0.11). It was also poor between the APAAP and the functional assay (κ=0.05) but fair between both flow cytometric assays (κ=0.24).

Discussion

Previously, we reported the results of P-gp detection in childhood ALL by immuno-cytochemistry and concluded that this technique was a useful tool to predict relapse [8]. In general, we demonstrated the good reproducibility, the low inter-observer variation and the high sensitivity of the immunocytochemical APAAP assay. This technique allows the evaluation of individual cells. In addition, it allows the discrimination between malignant and normal cells and the detection of tumour cell heterogeneity. Nevertheless, the French workgroup on P-gp detection does no longer recommend immunocytochemistry [17]. In general, flow cytometry is preferred to evaluate MDR in acute leukaemia [3,17]. This technique has the advantage of being independent of subjective interpretation. Large numbers of cells can be analysed and various P-gp expressing subpopulations can be recognised using specific markers in multiparameter tests. Flow cytometry is fast, reproducible and easy to perform. The functional assays are, in contrast to the immunological tests, independent of MoAb usage. Consequently, they are cheaper and not influenced by epitope masking. The main drawback of these flow cytometric assays is the need of viable cells. Moreover, the technique does not allow visual examination of the cells.

P-gp activity was less frequently observed in ALL than in ANLL, which is in accordance with published results [16,18]. There was no significant difference between both groups using immunological tests. In both ALL and ANLL, we found no significant increase in P-gp expression or activity at relapse. This finding is in accordance with the results published by Kanerva [14] but in contrast with the results of Ivy and Tafuri [11,20]. The latter observed an increased P-gp activity at relapse. Neither of them observed an increased P-gp expression.

Table 3: Results of P-gp expression using an immunocytochemical (IC) and flow cytometric (FC) immunological test and of the functional assay (Efflux)

	Total	IC-/FC- Efflux-	IC-/FC- Efflux+	IC+/FC+ Efflux-	IC+/FC+ Efflux+	IC+/FC- Efflux-	IC+/FC- Efflux+	IC-/FC+ Efflux-	IC-/FC+ Efflux+
De novo ALL	59	22	3	5	3	12	1	9	4
Relapse ALL	22	11	1	1	1	4	2	1	1
De novo AML	17	4	1	2	2	2	1	2	3
Relapse AML	7	1	3	1	1	0	0	1	0
Total	105	38	8	9	7	18	4	13	8

IC -: immunocytochemical test results are negative for both MoAbs
IC +: immunocytochemical test is positive for 4E3 or MRK16
FC -: immunological flow cytometric assay is negative for both MoAbs
FC +: immunological flow cytometric assay is positive for 4E3 or MRK16
Efflux -: there is no reversible Rho123 efflux; Efflux +: there is a reversible Rho123 efflux

Samples of 9 ALL and 6 ANLL patients were evaluated for P-gp expression and/or activity at diagnosis and at relapse, using both flow cytometric assays. Surprisingly, induction of P-gp expression at relapse was observed in only one ALL and one ANLL patient. Even the disappearance of initial P-gp expression or function was noticed. This is in accordance with the findings of van den Heuvel-Eibrink [22].

Between the different assays, poor correlations were observed. Eight patients with immunologically negative results, showed a reversible efflux. The sensitivity of both immunological assays was low due to false negative results. These findings suggest the presence of other, non-P-gp efflux pumps such as the members of the multidrug resistance associated protein family (MRP 1-7) or lung cancer related protein (LRP) [19]. The fluorescent dye used in this study, Rho 123, is also a substrate for MRP1 although it is less efficiently transported compared to P-gp [3]. In nine patients a positive immunological test result was found together with a negative functional assay. This may be caused by non-specific binding of the MoAbs or by the presence of non-active P-gp [15,23].

In this study, we demonstrated that both immunocytochemistry and flow cytometry can be used to detect P-gp expression in childhood acute leukaemia. We also conclude that P-gp expression is rather low in childhood acute leukaemia. In addition, in positive samples obtained from these patients, only small numbers of P-gp expressing cells are present. This stresses the importance of specific and sensitive detection techniques. We believe that immunocytochemistry, as well as flow cytometry, meet those requirements. In a prospective study (unpublished results), patients at risk for relapse could only be identified by combining the results of the immunocytochemical and the functional flow cytometric assay. Consequently, we recommend immunocytochemistry in combination with a functional flow cytometric assay to study P-gp expression and activity in childhood acute leukaemia.

Acknowledgments

The authors thank Jeannine Geerinck and Brendy Van Butsel for the excellent technical assistance. This work was supported by the Flemish Institute for the Promotion of Scientific Technological Research in Industry (I.W.T) and the Fund for Scientific Research (F.W.O. Vlaanderen).

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REPETITIVE ANALYSES OF P-GLYCOPROTEIN IN CHRONIC MYELOID LEUKAEMIA

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Acta Clinica Belgica 2000; 55: 215-221

REPETITIVE ANALYSES OF P-GLYCOPROTEIN IN CHRONIC MYELOID LEUKAEMIA

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Key-words: MDR, P-glycoprotein, chronic myeloid leukaemia, flow cytometry

ABSTRACT

P-glycoprotein, a pump located in the plasma cell membrane, extrudes several clinically important drugs from the cell, and hence causes multidrug resistance. Reversing clinical drug resistance is possible by using agents that inhibit the activity of P-glycoprotein. We describe the results of sequential flow cytometric determinations of P-glycoprotein expression and activity in two patients suffering from acute lymphoblastic transformation of chronic myeloid leukaemia. Neither P-glycoprotein expression, nor its activity could be detected in the initial sample of the first patient. In the second patient, no P-glycoprotein expression was found at diagnosis. However, after chemotherapy containing P-glycoprotein substrates, a significant expression was found in both patients and the functional flow cytometric test was positive. In order to achieve an accurate selection of patients that might benefit from the clinical use of P-gp inhibitors, repeated analyses are indicated in each patient suffering from acute leukaemia, during the course of the illness.

INTRODUCTION

Drug resistance of malignant cells, limiting successful chemotherapy, is known to arise through a variety of mechanisms (1). One of the best studied causes of multidrug resistance (MDR) is the energy-dependent efflux of drugs mediated by P-glycoprotein (P-gp), a pump located in the plasma cell membrane. This protein, encoded by the MDR1 gene, probably has a physiological function in detoxification of cells and secreting mechanisms of normal cells (2). In malignant cells, it extrudes several clinically important drugs with different chemical structures and intracellular targets, such as anthracyclines, vinca alkaloids and epipodophyllotoxins. P-gp may be present in malignant cells before any treatment or its expression may develop by exposure to certain chemotherapeutic agents (3).

Numerous studies have evaluated the role of P-gp expression as prognostic factor. In adults with acute myeloid leukaemia (AML), it was convincingly demonstrated that P-gp expression is a predictor of poor outcome (4,5). Its role in acute lymphoblastic leukaemia (ALL), both in adults and in children, remains more controversial (6,7). Chronic myeloid leukaemia (CML) is a well known example of a disease refractory to various chemotherapeutic agents, including some which are pumped out by P-gp. In general, P-gp overexpression is rarely detected at the chemotherapy responsive stages of CML, but more often at the time of blast crisis (8-15). Hence, P-gp may possibly affect therapeutic outcome in some cases of CML.

Phase I/II clinical trials using P-gp inhibitors have been conducted recently and yielded encouraging results (16-19). Usually, patients are classified at initial diagnosis using sets of biologic parameters identifying those likely to achieve a complete remission with conventional therapeutic regimens, as well as those unlikely to do so. However, we believe that in malignancy in general, and especially in patients with CML, only repeated analyses of MDR during the course of the illness will accurately identify patients with P-gp positive malignancy and hence, are indicated in order to select patients that might benefit from the clinical use of P-gp inhibitors. This is illustrated beneath.

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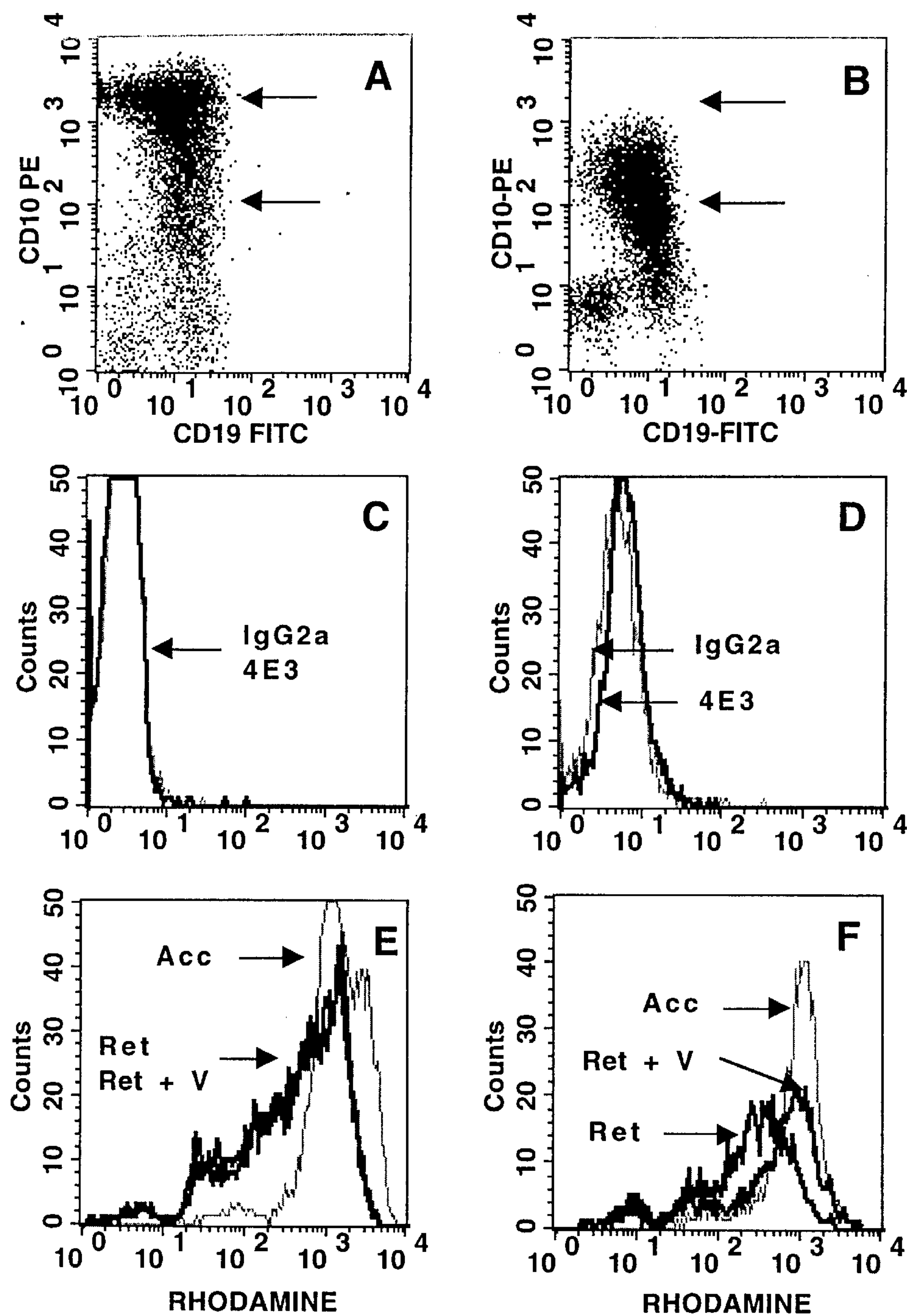

Figure 1 — Results of the immunophenotyping and of the immunological and functional P-gp evaluation at initial presentation (A, C, E) and after three weeks induction chemotherapy (B, D, F) in patient 1. A-B: Dot plots of CD10 versus CD19 revealing subpopulations with different CD10 staining intensity (arrows). C-D: Histograms with staining intensity of the anti-P-gp monoclonal antibody 4E3 (black line) compared to the isotypic matched control IgG2a (grey line). E-F: Histograms with intracellular rhodamine accumulation (light grey line) and retention in the absence (black line) and presence (grey line) of verapamil (V).

Table 1 — Sequential results of the flow cytometric immunological and functional test in an adult (patient 1) and a girl (patient 2) suffering from lymphoid blast crisis of CML

	Sample:		T0[d]	T0+3w[e]	T0+8w	TR[f]
Patient 1:	**blood:**	*MFI[b]:*	**1.06**	**1.16**	**1.11**	
		Rho RR[c]:	**0.98**	**2.28**	**2.65**	
	BM[a]:	*MFI:*			**1.41**	
		Rho RR:			**9.06**	
Patient 2:	**blood:**	*MFI:*	**0.81**			
		Rho RR:	**1.47**			
	BM:	*MFI:*	**0.87**			**1.23**
		Rho RR:	**2.17**			**1.43**

a: BM: bone marrow
b: MFI: mean fluorescence index (= mean fluorescence of cells stained with 4E3 divided by mean fluorescence of cells stained with IgG2a)
c: Rho RR: Rho 123 retention ratio (= median Rho 123 retention in the presence of verapamil divided by median Rho 123 retention in the absence of verapamil)
d: T0: initial diagnosis of blast crisis
e: T0+3w: 3 weeks after initial diagnosis
f: TR: relapse

PATIENT 1

Clinical history, haematological and cytogenetic evaluation:

A 55 year old man was admitted to the department of Haematology with a flu-like syndrome. Two years earlier, the diagnosis of a Philadelphia (Ph) chromosome positive CML was made. Treatment with hydroxyurea was started for cytoreduction, and after a haematological remission was achieved, interferon α-2a was started to consolidate the chronic phase and to induce a cytogenetic response.

Peripheral blood analysis showed a leukocytosis of 72 × 10^9/l, with 84% blasts, a thrombocytosis of 709 × 10^9/l and a normal haematocrit. The same blast population was found in the bone marrow (BM), reaching up to 80% of all nucleated cells. Morphologically, the blasts showed no granules or Auer rods. Myeloperoxidase staining, periodic acid-Schiff (PAS) and the acid phosphatase tests were negative. Only rare positivity was seen with Sudan black staining (<3% of the blasts).

Cytogenetic studies showed the known Ph chromosome with additional abnormalities, namely: 45,XY, del(7)(p13), t(9;22)(q34;q11), –20. Fluorescent in situ hybridisation (FISH) was performed to find the BCR/ABL rearrangement (using the LSI BCR/ABL 2-colour-translocation probe, Vysis, 32-190022). Co-localisation of ABL and BCR was found in 66% of the evaluated cells.

Immunophenotyping and molecular analysis:

Two-colour flow cytometric immunophenotyping was performed on BM, collected in EDTA tubes. Fifty µl aliquots were incubated for 15 minutes at room temperature with combinations of optimally titrated monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE).

Fluorescence was analysed on a FACSort flow cytometer (Becton Dickinson, Erembodegem, Belgium) calibrated with CaliBRITE™ beads. At least 10,000 events were counted and analysed with Cell Quest software.

The results of the immunophenotyping of the BM were: 90% CD10/TdT double positive, 62% CD10/CD19 double positive (Fig. 1A), 83% CD34/HLA DR double positive, 81% CD22 positive, and weak myeloid expression of CD33 (40%) and CD13 (36%). Other B-cell markers such as CD20 and CD21, the myeloid marker CDw65 and all T-cell markers (CD2, CD3, CD7) scored below 5% and were considered negative.

The clonality of the cells was confirmed by a polymerase chain reaction (PCR) analysis of the immuno-

globulin heavy chain (IgH) gene complementarity determining region 3. Therefore a consensus primer against the framework 3 region or framework 2 region was used in combination with a consensus primer against the J_H region. Both IgH PCRs were positive.

In accordance with the recommendations described by Van Bockstaele et al. (20), the diagnosis of a precursor B ALL with aberrant myeloid marker expression was made.

Flow cytometric detection of P-gp expression and functional activity:

Mononuclear cells were isolated by gradient centrifugation on Ficoll-hypaque.

Samples of the human myeloid leukaemia cell line K562 and its P-gp expressing resistant clone K562/VLB were included in each experiment as negative and positive controls, respectively.

1. P-gp expression:

Cells (5 x 10^5) were incubated for 30 minutes with a matched-isotype control antibody IgG2a (Dako Corporation, Glostrup, Denmark) and with the monoclonal antibody 4E3 (7.5 mg/l, Dako Corporation, Carpinteria, USA) which recognises an external epitope of P-gp. Cells were washed twice in PBS/BSA/NaN_3 and incubated for 20 minutes with FITC-labeled goat $F(ab')_2$ anti-mouse IgG (Caltag Laboratories, Burlingame, UK). Cells were stained with CD10 to identify the cell population of interest.

A sample was considered positive if the mean fluorescence of the cells stained with 4E3 was at least 10% brighter than the mean fluorescence of the cells stained with IgG2a, resulting in a mean fluorescence index (MFI) of at least 1.10 (21).

2. P-gp functional activity:

According to Ludescher et al. (22), 10^5 cells were incubated for 1 hour at 37°C with 200 µg/l rhodamine 123 (Rho 123, Sigma, St. Louis, USA), which is a P-gp substrate, in the absence and presence of 10 µM of the P-gp inhibitor verapamil (Knoll AG, Ludwigshafen, Germany). The intracellular Rho 123 accumulation was measured. Cells were washed twice with cold (4°C) HBSS without phenol red and resuspended. Cells were incubated for 1 hour at 37°C with or without 10 µM verapamil and the remaining intracellular Rho 123 retention after the efflux period was measured. Results were expressed as ratios which are calculated after the accumulation or efflux period of 1 hour each, by division of the median intracellular Rho 123 fluorescence in the presence of verapamil by the median Rho 123 fluorescence in the absence of verapamil. The functional assay was considered positive if the cells exhibited Rho 123 efflux and if the intracellular Rho 123 fluorescence enhanced in the presence of verapamil by at least 10%, resulting in a Rho 123 retention ratio (RR) of at least 1.10 (21).

The immunological assay using the monoclonal antibody 4E3 was negative in this patient (MFI = 1.06, Table 1) since there was no difference in cellular fluorescence of the isotypic antibody IgG2a and 4E3 (Fig. 1C). In the functional test, after comparison of the Rho 123 retention (Fig. 1E, black line) with the Rho 123 accumulation (Fig. 1E, light grey line), we concluded that efflux was present. However, there was no influence of the P-gp inhibitor verapamil on this efflux (Fig. 1E, grey line) and the Rho 123 RR was only 0.98 (Table 1).

Follow-up:

The patient was treated according to the ALL scheme of Hoelzer (5/93) for adults. Induction chemotherapy consisted of corticosteroids, daunorubicin, vincristine and asparaginase. The peripheral WBC number gradually decreased (Fig. 2). At day 15 and day 21, the number of blasts in the peripheral blood was determined and was 73% and 89% of the total WBC count, respectively.

At day 21, immunophenotyping was repeated to identify the remaining blastic population in the peripheral blood. A distinct phenotypic shift was observed: the blasts no longer expressed CD34 nor HLA DR; 81% of the cells were CD10/TdT double positive, and 78% were CD10/CD19 double positive. A weak CD33 expression was still observed. Whereas in the initial sample all cells

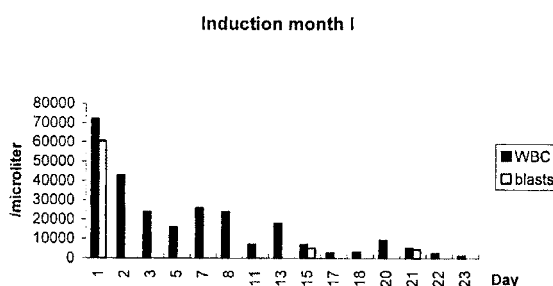

Figure 2 — WBC and blast count during induction therapy (patient 1).

were CD19 positive, two cell populations could be discriminated with different CD10 staining intensity (Fig. 1A, arrows). After chemotherapy, the blasts were CD19 positive but only the weakly CD10 positive subpopulation remained (Fig. 1B, arrows).

In this sample, three weeks after starting chemotherapy, a weakly positive 4E3 staining was found (Fig. 1D, black line) compared to the isotypic antibody (Fig. 1D, grey line) with a MFI of 1.16 (Table 1). The blasts exhibited Rho 123 efflux, which was clearly reversed by verapamil (Fig. 1F) and hence resulted in a Rho 123 RR of 2.28 (Table 1). P-gp expression and activity were also determined in blood and BM blasts 8 weeks after initial diagnosis and remained positive (Table 1). The patient remained refractory to treatment and eventually died 5 months after the diagnosis of blast crisis.

PATIENT 2:

Clinical history, haematological and cytogenetic evaluation:

In a 10 year old girl, presenting with bone pain, the diagnosis of CML was made. Peripheral blood analysis showed a leucocytosis of 90 x 10^9/l, with 73% blasts, and 833 × 10^9/l platelets. In the BM 76% blasts were found, morphologically without granules or Auer rods. PAS staining and acid phosphatase tests were negative; 4% of the blasts had a positive myeloperoxidase staining. Cytogenetic evaluation revealed the presence of a Ph chromosome (46,XX, t(9;22)(q34,q11)) which was confirmed by FISH analysis. Both BM and cytogenetic abnormalities were consistent with CML of the adult type.

She was initially treated with hydroxyurea and interferon α-2 in the chronic phase of her disease. Six months after initial diagnosis, she developed a blast crisis.

Immunophenotyping:

Immunophenotyping was perfomed on BM as described previously. The blasts were positive for the B-cell markers CD19 (75%), CD22 (71%) and CD10 (64%). They were negative for the T-cell markers CD2 (9%), CD7 (7%), cyCD3 (7%) and positive for the myeloid markers CD13 (90%) and CD33 (82%). Other positive markers were CD34 (69%), TdT (76%) and HLA-DR (89%). Hence, the diagnosis of a precursor B ALL with aberrant myeloid marker expression was made. The clonality of cells was not confirmed by molecular analysis.

Flow cytometric detection of P-gp expression and functional activity:

This child was also evaluated for P-gp presence by means of the flow cytometric assays as described above and the results are presented in table 1. At the initial diagnosis of lymphoid blast crisis, no P-gp expression was found (MFI of 0.81 and 0.87 in blood and BM, respectively) whereas P-gp activity was clearly positive, both in BM (RR=2.17) and in blood leukaemic cells (RR=1.47).

Follow-up:

The girl received multichemotherapy containing idarubicin, cytosine arabinoside and etoposide, which is a P-gp substrate. She did not reach a complete remission and underwent a bone marrow transplantation (BMT) with BM of a matched unrelated donor. The conditioning regimen comprised total body irradiation and the administration of cycloblastine. Anti-thymocytes globulin was employed to prevent graft rejection. Graft versus host profylaxis consisted of cyclosporin A and methotrexate. The immediate posttransplant period was uneventful and the patient experienced complete recovery of haematopoiesis. She left the hospital ten weeks after transplantation in complete remission. Five months after BMT, she relapsed and the blasts in BM and peripheral blood showed the same immunophenotype and cytogenetic abnormalities. At this relapse after BMT, the blasts exhibited a strong P-gp expression (MFI=1.23) and the functional test remained positive (RR=1.43) (table 1). No new induction treatment was administered and the girl died three months later.

DISCUSSION

Several agents, including cyclosporin A and PSC833, can reverse the MDR phenotype at concentrations that can be achieved in vivo (17-19,23). Since P-gp reverting agents have side effects and may increase the toxicity of cytostatic agents by inhibiting P-gp in the liver and the kidneys of the patient, accurate identification of patients that will benefit from reverting agents seems indispensable.

Nowadays, P-gp expression and activity can easily be detected in vitro using sensitive and specific flow cytometric assays (24). Flow cytometry seems to be

clearly superior to immunocytochemistry and reverse transcriptase-PCR in distinguishing specimens with low or intermediate levels of P-gp expression; the Rho 123 efflux test equally detects low levels of drug resistance (25). Moreover, flow cytometric functional assays can help to evaluate the in vitro effect of chemosensitizers on leukaemic blasts.

As demonstrated in these case reports, not only diagnostic samples but also follow-up samples should be screened for P-gp presence. In the initial sample of patient 1, Rho 123 efflux was observed, without effect of verapamil. After induction chemotherapy, containing the P-gp substrates vincristine and daunorubicin, a weak but significant P-gp expression was seen and the reverting effect of verapamil on Rho 123 efflux was clearly demonstrable. For patient 2, the immunologic assay only scored positive at relapse, whereas it was negative at initial diagnosis.

Literature data on the frequency of P-gp expression in different CML stages are variable, especially with respect to the chronic phase of the disease (15). This is probably due to the small numbers of patients included in some studies, the use of different detection assays and thresholds, and the lack of evaluating P-gp activity since inactive P-gp seems to be exclusively found in the chronic and accelerated phases of CML (14,15). P-gp was never analysed in our patients during the chronic phase of their CML.

In general, P-gp expression is found more frequently in accelerated and blastic phase CML cases (9,12-15). It has been suggested that this elevated expression in blast crisis reflects the enrichment of immature myeloid cells, which intrinsically express P-gp (10). This was not the case in our first patient since P-gp was detected in the cell population which had lost its CD34/HLA DR expression.

Sequential examinations of CML patients were performed in several studies. Stavrovskaya et al. (15) found some P-gp positive cases that became negative during progression from the chronic phase to blast crisis. They also studied 14 patients sequentially during the course of a blast crisis, in which they received polychemotherapy containing drugs known to select for MDR. An increase in the fraction of P-gp positive cells was found in 2 of 9 initially negative patients, whereas 4 of 5 initially positive patients became negative on a second examination. Turkina et al. (14) evaluated 11 patients in blast crisis consecutively during treatment using an immunological assay and found an enhancement in P-gp expression in only 2 patients. Since all these 11 patients were highly refractory to chemotherapy, the authors concluded that P-gp mediated resistance only plays a role in particular cases of CML. The same conclusions were drawn by Weide et al. (9). On the contrary, in the study of Kuwazuru et al. (8) 3 of 11 patients in blast crisis became positive at relapse and were clinically resistant to therapy. These authors concluded that either P-gp expression is enhanced or the cells expressing P-gp are favoured during chemotherapy and that P-gp expression is closely associated with drug-resistance in CML. Similar results were obtained by others (11,12,16).

In the other haematological malignancies, Ma et al. (26) reported for the first time sequential P-gp detections and they observed in two patients with AML an increase in the staining intensity and in the proportion of P-gp positive cells during progression of the disease and the clinical development of drug resistance. Meanwhile, several studies have shown that treatment of leukaemic patients with anthracyclines and vinca-alkaloids may further increase P-gp expression in (previously negative) patients or that the expression levels are higher in previously treated and/or refractory patients (11,18,27). However, it is unclear whether the MDR resistance phenotype is induced by exposure to certain drugs or whether sensitive and resistant cell populations are simultaneously present at the start of treatment. The immunophenotypic results of our first patient favour the hypothesis that there were at least two different leukaemic clones present at initial diagnosis. We assume that the number of drug-resistant cells in the original sample was too low to detect P-gp by the flow cytometric tests. At day 21, when the predominant clone had disappeared from the peripheral blood, we could clearly demonstrate P-gp activity.

In conclusion: We emphasize the importance of repeated P-gp determinations during the treatment of leukaemic patients, and in particular patients suffering from CML in blast crisis, in order to select those patients that might benefit from the clinical application of P-gp reverting agents.

SAMENVATTING

P-glycoproteïne, een efflux-pomp gelegen in de plasma membraan, verwijdert cytotoxische medicatie vanuit het cytoplasma naar het extracellulair milieu en geeft zo aanleiding tot het ontstaan van resistentie. Opsporen van P-glycoproteïne is klinisch relevant aangezien inhibitoren ervan kunnen toegevoegd worden aan de chemotherapie. Wij rapporteren de resultaten van opeenvolgende flowcytometrische bepalingen van de expressie en de activiteit van P-glycoproteïne bij

twee patiënten wiens chronische myeloïde leukemie transformeerde naar een acute lymfoblasten leukemie. Noch expressie, noch activiteit van P-glycoproteïne werden gedetecteerd in het diagnostisch staal van de eerste patiënt. Bij de tweede patiënt werd initieel géén P-glycoproteïne expressie teruggevonden. Na chemotherapie echter werden beide positief. Om leukemie patiënten die in aanmerking komen voor een behandeling met P-glycoproteïne inhibitoren nauwkeurig te selecteren, zijn herhaalde bepalingen over het verloop van de ziekte onontbeerlijk.

ACKNOWLEDGEMENTS

The authors thank Prof. Dr. J. Delanghe and Dr. A. Verstraete for their helpful remarks.

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5 Scintigrafische functionele test

Technetium-99m hexakis 2-methoxyisobutylisonitrile (^{99m}Tc sestamibi) is een lipofiel en kationisch radiofarmaceutisch product. Men vermoedt dat ^{99m}Tc sestamibi passief op basis van een electrochemische gradiënt doorheen de celmembraan diffundeert en accumuleert in de mitochondria van de cel (Figuur 4, pagina 66) (Piwnica-Worms et al., 1990; Crane et al., 1993).
^{99m}Tc sestamibi wordt snel uit het bloed geklaard en wordt bij voorkeur opgenomen in cellen rijk aan mitochondria, zoals het hart en maligne tumoren. Ook de opname door de nier is zeer sterk. ^{99m}Tc sestamibi opname gebeurt ook in vele andere organen: speekselklieren, schildklier, hart, lever, galblaas en darmen (hepatobiliaire secretie). Er wordt slechts een minimale opname gezien in het beenmerg en er is geen opname in spierweefsel. Behalve de hepatobiliaire secretie is er ook een urinaire secretie. De “wash-out” vanuit het myocard is over een verloop van 4 uur tijd zeer beperkt. De halfwaardetijd van het isotoop bedraagt 6.6 uur (Abdel-Dayem et al., 1994).

^{99m}Tc sestamibi werd voor het eerst toegepast in de kliniek bij perfusie-onderzoek van het myocard als alternatief voor Thallium (^{201}TI) (Wackers et al., 1989). In 1987 ontdekte Muller et al. tijdens een cardiaal onderzoek de opname van ^{99m}Tc sestamibi in een longtumor. Hierna werd ^{99m}Tc sestamibi beeldvorming uitgebreid nagekeken als detectiemethode voor tumoren. De opname in longtumoren, borsttumoren, lymfomen, osteosarcomen, ongedifferentieerde mesenchymale tumoren, en tumoren van de schildklier werd reeds beschreven (overzicht door Hendrikse et al., 1999).

Omdat ^{99m}Tc sestamibi een substraat blijkt te zijn voor zowel P-gp als MRP1, werd het toegepast om in vivo MDR na te kijken (Piwnica-Worms et al., 1993; Hendrikse et al., 1998). Als niet-invasief onderzoek opende ^{99m}Tc sestamibi veel perspectieven om tumoren te screenen bij diagnose of tijdens de behandeling om uiteindelijk een prognostische stratificatie van de patiënten toe te laten. Beeldvorming tijdens de toediening van modulatoren zou bovendien in vivo kunnen aantonen bij welke patiënten modulatoren effect hebben (Chen et al., 1997; Bakker et al., 1999).

Er werden reeds verschillende studies gepubliceerd waarin ^{99m}Tc sestamibi beeldvorming gecorreleerd werd met de resultaten van P-gp bepalingen (Moretti et al., 1996; Del Vecchio et al., 1997; Kostakoglu et al., 1997 en 1998). Slechts twee studies beschreven ^{99m}Tc sestamibi scans bij kinderen met een stadium 4 neuroblastoom. In deze studies werden conclusies over MDR getrokken, zonder in vitro P-gp of MRP1 te bepalen (Özcan et al., 1999; Burak et al., 1999).

6 Discussie

In dit onderzoek werden gevoelige en specifieke detectietechnieken van P-gp ontwikkeld, zodat leukemiestalen en stalen afkomstig van neuroblastaire tumoren onderzocht konden worden.

Tot nu toe is de diagnose van P-gp gemedieerde MDR in vaste tumoren en leukemieën vooral gebaseerd op in vitro testen. Wij legden de nadruk op de detectie van het eiwit zelf met de bedoeling zowel de eiwitexpressie als de activiteit van P-gp na te gaan.
Er werd géén MDR1 mRNA opgespoord. Post-transcriptionele veranderingen (bijvoorbeeld fosforylaties) aan P-gp zullen immers discrepanties tussen eiwit en mRNA expressie veroorzaken.
Bovendien werden geen "bulk"-technieken gebruikt omdat hierin geen onderscheid tussen kankercellen en normale stromale cellen gemaakt kan worden en omdat bovendien de informatie over heterogeniteit in de expressie verloren gaat. Kleine P-gp positieve subpopulaties in de tumor die kunnen aanleiding geven tot klinische MDR zullen immers niet gedetecteerd worden.

Als uitgangspunt werd door ons de immuuncytochemische APAAP techniek gebruikt, zoals die reeds vroeger beschreven werd (Dhooge et al., 1994). Het grote voordeel van deze test is een rechtstreekse, individuele evaluatie van elke cel. Maligne cellen kunnen van normale cellen onderscheiden worden. Bovendien werd aangetoond dat deze test gevoelig is in ALL op kinderleeftijd en een behoorlijke reproduceerbaarheid heeft (Dhooge et al., 1999). Het grootste nadeel van de immuuncytochemische techniek is een subjectieve interpretatiemogelijkheid. Vooral daarom wordt immuuncytochemie niet meer aanbevolen als enige detectietechniek in klinische studies (Marie et al., 1997).

Verschillende onderzoekers verkiezen flowcytometrische immunologische testen om P-gp te detecteren in leukemiestalen (Broxterman et al., 1997). Deze testen zijn objectief, heel goed reproduceerbaar en zeer gevoelig, vooral wanneer multiparametrische testen uitgevoerd worden.
Functionele flowcytometrische testen geven daarenboven klinisch relevante informatie. Sterk fluorescente kleurstoffen zoals Rho 123 worden immers door P-gp getransporteerd en kunnen flowcytometrisch gedetecteerd worden. In dit onderzoek werd een gevoelige en specifieke functionele test oppuntgesteld, waarin de Rho 123 concentratie in de leukemische cellen gemeten werd in de aan- en afwezigheid van de P-gp inhibitor verapamil.

Omdat er vaak discordanties teruggevonden worden tussen de flowcytometrische immunologische en functionele testen (Leith et al., 1995; Xie et al., 1995) wordt aanbevolen om beide testen uit te voeren op hetzelfde staal.
In ons onderzoek observeerden we discrepante resultaten tussen immunologische en functionele testen bij leukemiestalen van kinderen (Swerts et al., *submitted*). Ook tussen de immuuncytochemische en immunologische flowcytometrische test werden discordanties teruggevonden. Uit onze resultaten besloten we dat de immuuncytochemische APAAP techniek en de functionele flowcytometrische test additionele informatie geven en dus best samen worden uitgevoerd en geïnterpreteerd.

Bovendien toonden we aan dat zowel P-gp expressie als functie niet enkel in diagnostische stalen, maar ook in stalen tijdens de behandeling bepaald moeten worden (De Moerloose et al., 2000). Dit is vooral belangrijk wanneer overwogen wordt om aan patiënten P-gp modulatoren toe te dienen. In deze studie konden we daarenboven aantonen dat er bij diagnose van de blastencrisis van een volwassen patiënt met CML minstens twee immuunfenotypisch verschillende klonen aanwezig waren. Tijdens de chemotherapie werd deze patiënt klinisch resistent en prolifereerde één van deze klonen. In deze resistente kloon werd P-gp expressie en functie teruggevonden.

In onze studie van kinderen met leukemie (Swerts et al., *submitted*) waren er 9 ALL en 6 ANLL patiënten die bij diagnose onderzocht werden en eveneens bij recidief. Alle recidieven traden op nadat de behandeling reeds beëindigd was. Slechts twee van deze 15 kinderen werden flowcytometrisch P-gp positief bij recidief. Bij enkele kinderen verdween de initiële positiviteit zelfs bij recidief! Het is onduidelijk of dit te wijten is aan down-regulatie van P-gp expressie na het stoppen van de behandeling (Vanden Heuvel-Eibrinck, 2000).

De flowcytometrische immunologische en functionele testen werden aangepast om ook in stalen van neuroblastoompatiënten P-gp expressie en functie te kunnen nagaan. Hierop wordt echter ingegaan in hoofdstuk 4.

We ontwikkelden in dit onderzoek een scintigrafische functionele test voor P-gp met behulp van het isotoop ^{99m}Tc sestamibi. De voordelen van dit in vivo functioneel onderzoek van P-gp zijn velerlei: het onderzoek is gemakkelijk uit te voeren, het is niet invasief en het is onafhankelijk van biopten of resectiemateriaal. Het ^{99m}Tc sestamibi beeldvormingsonderzoek kan gemakkelijk op verschillende tijdstippen tijdens de behandeling uitgevoerd worden en zo relevante informatie geven. De resultaten van dit onderzoek worden verder besproken in hoofdstuk 4.

HOOFDSTUK 3

P-GLYCOPROTEÏNE EXPRESSIE EN FUNCTIE BIJ KINDEREN MET ACUTE LYMFOBLASTEN LEUKEMIE

1 Expression of the multidrug transporter P-glycoprotein is highly correlated with clinical outcome in childhood acute lymphoblastic leukemia: results of a long-term prospective study.

C. Dhooge, B. De Moerloose, G. Laureys, A. Ferster, D. De Bacquer, J. Philippé, J. Leroy, Y. Benoit

Leukemia and lymphoma 2002; 43: 309-314

2 The combined analysis of P-glycoprotein expression and activity predicts outcome in childhood acute lymphoblastic leukemia.

B. De Moerloose, K. Swerts, Y. Benoit, G. Laureys, T. Loeys, J. Philippé, C. Dhooge

Pediatric Hematology and Oncology, *submitted*

3 **Discussie**

EXPRESSION OF THE MULTIDRUG TRANSPORTER P-GLYCOPROTEIN IS HIGHLY CORRELATED WITH CLINICAL OUTCOME IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA: RESULTS OF A LONG-TERM PROSPECTIVE STUDY.

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Leukemia and Lymphoma, 2002, VOL. 43 (2), pp. 309–314

Taylor & Francis healthsciences

Expression of the Multidrug Transporter P-glycoprotein is Highly Correlated with Clinical Outcome in Childhood Acute Lymphoblastic Leukemia: Results of a Long-Term Prospective Study

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(In final form 1 July 2001)

The improved cure rate in childhood ALL may be attributed largely to the effective multidrug regimens currently applied in well-designed clinical trials. However, in a minority of patients with ALL, chemotherapy failure remains a leading cause of cancer related death, most probably due to cellular drug resistance. The better-known mechanism of such resistance is mediated by P-glycoprotein (P-gp). In a long term prospective study (mean time of follow-up: 65 months) the multidrug efflux pump P-gp was examined immunocytochemically in leukemic cells of 102 protocol-treated children with *de novo* acute lymphoblastic leukemia (ALL) and of 37 children with relapsed ALL. Fourteen percent expressed P-gp at initial diagnosis and 35% were P-gp positive at relapse. The patients being P-gp positive at initial diagnosis had a higher rate of leukemic relapse than the P-gp negative patients ($P = 0.02$). In the relapsing patients, those who were P-gp positive had a 2.18-fold greater risk for leukemic death than those who were P-gp negative. Paired analysis on diagnostic and relapsed samples from 20 patients did not support the hypothesis of P-gp mediated expression being a chemotherapy induced phenomenon. The cumulative event free survival for *de novo* ALL patients was significantly higher in the P-gp negative patient group. Multivariate analysis showed that P-gp expression is independent of other known risk factors. In conclusion we strongly advise that tests for P-gp in leukemic blasts should be conducted for every child with ALL, since this parameter selects a subgroup of patients with increased risk for leukemic relapse.

Keywords: P-glycoprotein; Multidrug resistance; Childhood leukemia; Immunocytochemistry

INTRODUCTION

The improved cure rate in childhood ALL may be attributed largely to the effective multidrug regimens currently applied in clinical trials [1]. However, in a minority of patients with ALL, chemotherapy failure remains a leading cause of cancer related death, most probably due to cellular drug resistance [2]. The better-known mechanism of such resistance is mediated by P-glycoprotein (P-gp) encoded by the multidrug resistance gene, *MDR*1 [3]. This transmembranous P-gp actively extrudes from the cell a variety of lipophilic compounds including amphipathic neoplastic agents. The net result is lower intracellular accumulation and hence reduced cellular toxicity of chemotherapeutics [4]. This decrease in chemosensitivity may be reversed by various modulating chemicals [5]. As some chemotherapeutic regimens are currently including P-gp modulating substance (Pediatric Oncology Group AML 9421 trial), knowledge on P-gp expression and its clinical implication in childhood ALL becomes a prerequisite. From the few prolonged follow-up observations reported [6–8], the impression arises that the ALL relapse rate is higher in patients with P-gp positive cell samples. Until now, the prognostic value of P-gp expression in childhood ALL has been unclear because of the following: the available data

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ISSN 1042-8194 © 2002 Taylor & Francis Ltd
DOI: 10.1080/10428190290006080

were obtained in a series of cell samples from pediatric as well as adult patients; the observations were pooled from ALL and ANLL patients; relapse as well as initial leukemia samples, mostly unpaired, were used in this data pooling [9–12]. We report the results of a prospective study conducted over 9 years, designed to examine whether expression of P-gp is of any prognostic value in childhood ALL.

PATIENTS AND METHODS

Two groups of patients were prospectively studied. The first consists of 102 consecutively (October 1990–September 1996) recruited children with *de novo* ALL, aged between 2 months and 16 years. The second group includes 37 patients with relapsed ALL (Fig. 1). In all patients the diagnosis of ALL was based on standard morphology, cytochemistry and immunophenotyping of the leukemic blasts. Data on response to 1 week corticosteroids, risk factor (RF) calculation [13], remission status, event free survival (EFS) and overall survival (OS) were available for all 102 patients. Successful karyotyping of the leukemic cells was conducted in 93 patients and cellular DNA content was assayed by flow cytometry in 68 patients. All patients were treated according to the European Organisation for Research and Treatment of Cancer-Childhood Leukemia Cooperative Group (EORTC-CLCG) 58881 protocol, including vincristine and anthracyclines. Bad corticosteroid response (bloodblasts > 1×10^9/L at day 8) and patients with unfavorable karyotype were treated with an intensified protocol. The frequency distribution of examined prognostic variables was not statistically different in the P-gp negative group or the P-gp positive group [8]. All patients were followed on a regular basis with the mean time of follow-up being 65 months (range 36–107 months, median: 64 months).

In 20 out of the 37 patients with leukemic relapse, samples at primary diagnosis were also available for P-gp expression (Fig. 1). The other 17 samples were from patients treated for primary ALL before initiation of the study. At first relapse (isolated bone marrow relapse in 20 children; extramedullar relapse in seven; combined relapse in ten) treatment was given according to the EORTC-CLCG protocols. Nineteen patients were treated

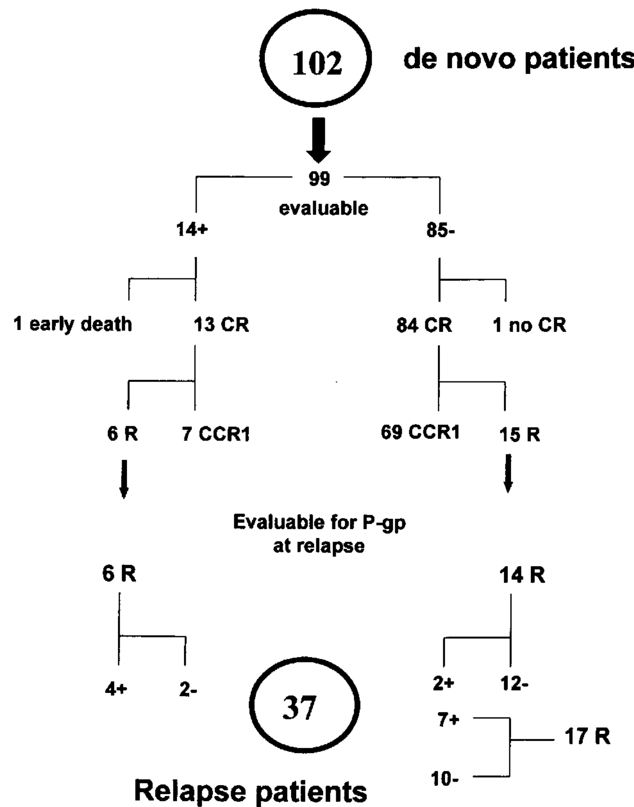

FIGURE 1 102 *de novo* ALL patients and 37 patients with a relapse of ALL were examined for P-gp expression. Of the 99 evaluable patients for P-gp at initial diagnosis, complete remission (CR) was obtained in 97 patients. Fourteen patients were P-gp positive, of which seven experienced an event (No CR (1); relapse (R) (6)). In 85 P-gp negative patients, an event was seen in 16 (No CR (1); R(15)). Samples for P-gp expression at relapse were available in 20 patients with a relapse during the study period and in 17 relapsed patients who had been treated for *de novo* ALL before the study period. CCR1: continuous first complete remission.

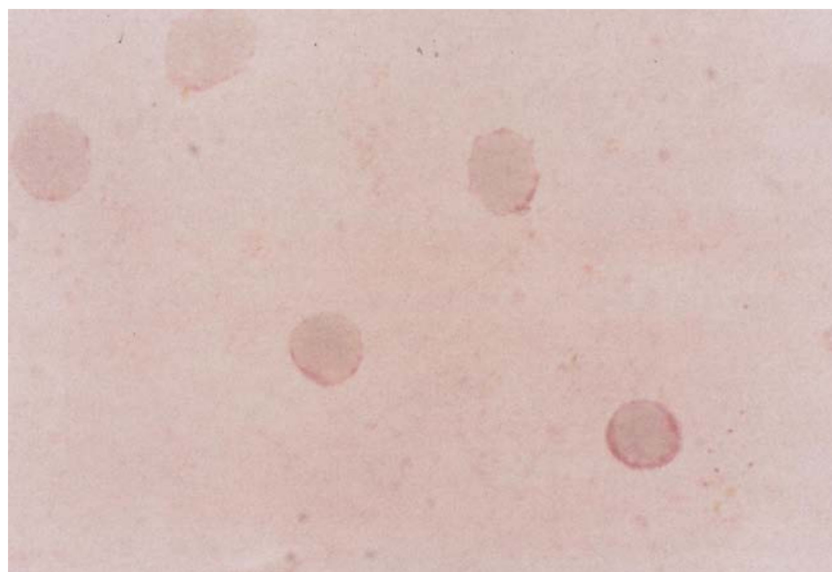

FIGURE 2 P-gp positive blasts in a BM smear of a child with ALL (APAAP with 4E3).

with chemotherapy alone, 17 underwent bone marrow transplantation (autologous transplant in one, allogeneic matched sibling transplant in six, matched unrelated donor transplant in ten). One patient was not given further therapy. Seven patients succumbed to treatment related toxicity, all being in complete remission (CR) at the moment of death.

Smears of peripheral blood and bone marrow were examined immunocytochemically for P-gp expression using the monoclonal antibodies (Mab) C219 and 4E3 as previously described [8,14] (Fig. 2). The human leukemia cell-lines K562 and K562/VLB, as well as patient slides without primary antibody, were consistently included as negative and positive controls. The results were interpreted independently by three observers. No arbitrary minimal cut-off limits were set, as has been proposed in workshops on MDR [15,16]. A given sample was termed P-gp positive when the Mab C219 treatment yielded a positive staining (Table I (1 Mab)). The latter was shown to be more sensitive than the Mab 4E3 in a previous study [8]. Three patients in whom only one slide was examined for P-gp with the Mab 4E3 only were not included in the statistical analysis of P-gp as a prognostic parameter. The same analysis was done with a patient defined P-gp positive when a positive staining was seen with both Mabs (Table I (2 Mabs)).

Statistical Methods

Cumulative event-free-survival (EFS) and mortality were estimated according to the Kaplan–Meier method. The relative impact on event (relapse or leukemic death) and on mortality of baseline variables was expressed as Risk Ratios (RR). Multivariate analysis was performed according to the Cox proportional hazard modeling method. Relapsed patients with fatal outcome due to other reasons than resistant leukemia were labeled as censored cased in the statistical analysis. The *a priori* probability level, $\alpha = 0.05$, was used for defining statistical significance.

RESULTS

Remission Status, Event Free Survival and Overall Survival

By induction treatment, CR was achieved in 100/102 (98%) children with *de novo* ALL. Two patients who did not achieve CR succumbed to the disease. No fatal outcome was noticed in patients in first complete remission (CR1). Nine years after the start of the study, 21/100 patients who had reached CR have relapsed (Fig. 1). In the total group of *de novo* ALL patients, EFS at 5 years was 77.1%, OS 85%. In the relapsed patient group mean time from initial diagnosis to first relapse was 32 months and median survival time 27 months after relapse.

Prognostic Value of P-gp Expression

1. *At initial diagnosis*: Fourteen of 99 (14%) children were found to express P-gp at the time of initial diagnosis. Seven of them experienced an adverse event whereas 16 of 85 P-gp negative patients ran a comparable unfavorable clinical course. Amount the risk factors examined by multivariate analysis, P-gp status was significantly associated with a risk for relapse ($P = 0.02$, Table I). Moreover this multivariate analysis has shown that P-gp status was independent of other risk factors, and as powerful a prognostic factor as either WBC count or age at

diagnosis. Figure 3 shows the difference in cumulative EFS according to P-gp expression in leukemic blasts at initial diagnosis. EFS was significantly longer in the P-gp negative group than in the P-gp positive patients (Log-rank $P = 0.005$—Fig. 3).

Only eight out of 99 patients with *de novo* ALL were scored P-gp positive with both Mabs. Once again, statistical analysis showed that P-gp expression was highly correlated ($P = 0.03$) with an increased relative risk of relapse (Table I (2 Mabs)).

2. *At relapse*: Thirteen among 37 samples of relapse ALL (35%) were scored positive for P-gp (Fig. 1). This is significantly higher than the P-gp positive rate at initial diagnosis ($P = 0.013$). Among the patients with relapse, nine out of 13 P-gp positive ones succumbed to their leukemia and one is alive with resistant disease. Such unfortunate outcome occurred in only eight of 24 P-gp negative relapsed patients. P-gp positive relapsed patients have a 2.18-fold greater risk for leukemic death than the P-gp negative relapsed patients (Table II).

3. *Does chemotherapy induce P-gp expression?* P-gp was found negative at initial diagnosis in 14 out of 20 patients with a leukemic relapse of whom paired samples could be included. This state was maintained in 12 of 14 patients at time of their first leukemic relapse.

Evaluation of Other Prognostic Factors

Among the risk factors examined in patients at initial diagnosis, multivariate analysis showed that P-gp expression, age and WBC count were independent prognostic factors for predicting relapse (Table I). Hyperdiploidy (more than 50 chromosomes), found in the leukemic blasts of 15 children, was not significantly associated with a better outcome. Relative DNA content between the indices of 1.16 and 1.6 found in 13 patients was of no prognostic consequence in this study.

In the patient group with leukemic relapse, a timespan of more than 30 months from initial diagnosis to the moment of relapse was clearly associated ($P = 0.02$) with a better outcome (Table II).

DISCUSSION

Although apparently infrequent in *de novo* childhood ALL, expression of P-gp in leukemic cells as immunocytochemically detected in this long term prospective study, is directly correlated with a higher risk for leukemic relapse. By prolonging the time of follow-up of the same patients by 17 months, the prognostic value of P-gp expression on cumulative EFS is even more significant than recorded in a previous report [8]. If termed P-gp positive only when reacting with both Mabs [15,16], the prognostic power of the P-gp score is judged also to be statistically significant. The overall findings confirm and extend the conclusions of other studies [6,17]. Goasguen and Dossot [6] reported a lower survival rate in 36 P-gp positive than in P-gp negative patients, and Tafuri *et al.* [17] found the mean rhodamine efflux value in 14 (either

TABLE I Event-free survival in patients according to different prognostic factors at initial diagnosis. RR_u: Univariate risk ratio (95% confidence interval). RR_m: Multivariately adjusted risk ratio (95% confidence interval) according to Cox proportional hazard modeling

Prognostic factors	% event	Event rate	RR_u	*P*	RR_m (1 Mab)	*P*	RR_m (2 Mab)*	*P*
Age								
1–10 years	18.4 (16/87)	27.9	1		1		1	
<1 or >10 years	46.7 (7/15)	142.0	3.60 (1.47–8.82)	0.005	2.93 (1.17–7.36)	0.02	2.63 (1.04–6.63)	0.04
Sex								
F	23.5 (12/51)	38.3	1					
M	21.6 (11/51)	35.5	0.93 (0.41–2.11)	0.86				
Immunophenotype								
B-cell lineage	21.4 (19/89)	34.2	1					
T-cell lineage	30.8 (4/13)	58.4	1.58 (0.54–4.66)	0.40				
WBC								
$\leq 100 \times 10^9$/L	18.7 (17/91)	29.1	1		1		1	
$> 100 \times 10^9$/L	54.6 (6/11)	154.2	4.42 (1.73–11.25)	0.002	3.43 (1.30–9.02)	0.01	4.11 (1.53–11.02)	0.005
Blasts day 8								
$\leq 1 \times 10^9$/L	20.6 (19/92)	32.7	1					
$> 1 \times 10^9$/L	33.3 (3/9)	70.6	2.01 (0.59–6.79)	0.26				
DNA index								
≥1.16 and ≤1.6	23.1 (3/13)	40.2	1					
<1.16 and >1.6	21.8 (12/55)	32.3	0.98 (0.28–3.46)	0.97				
Chromosomes								
Hyperdiploid	13.3 (2/15)	21.6	1					
Other+unfavorable	23.1 (18/78)	37.9	1.83 (0.42–7.89)	0.42				
P-gp at diagnosis (1 Mab)								
Negative	18.8 (16/85)	29.5	1		1			
Positive	50.0 (7/14)	112.5	3.31 (1.36–8.06)	0.008	3.24 (1.32–7.96)	0.01		
*P-gp at diagnosis (2 Mab)**								
Negative	20.9 (19/91)	33.3	1				1	
Positive	50.0 (4/8)	119.0	3.15 (1.07–9.27)	0.04			3.51 (1.15–10.71)	0.03

* P-gp positivity as defined by a positive staining for both Mabs.

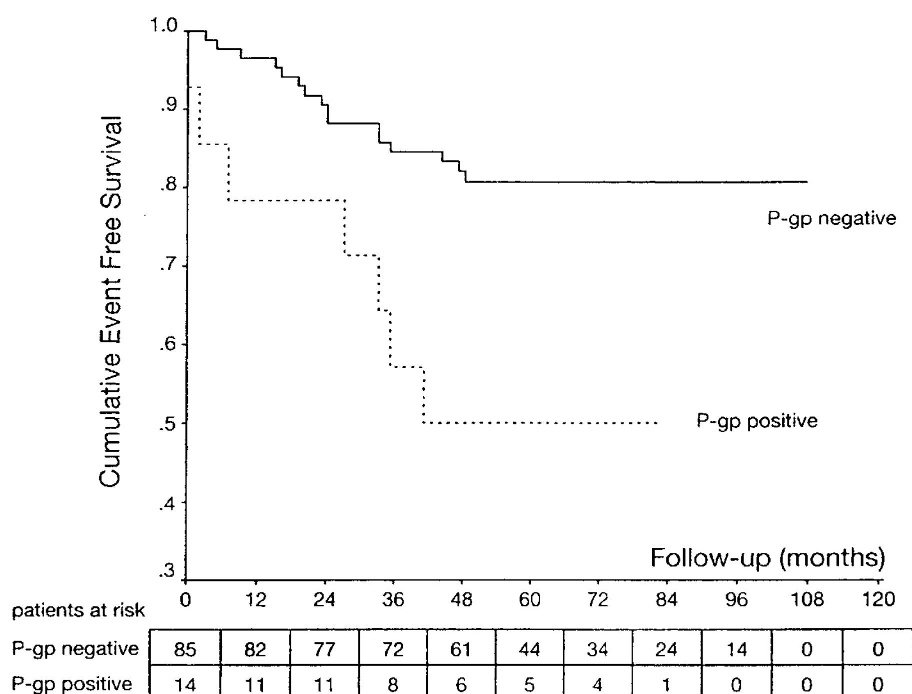

FIGURE 3 Kaplan–Meier cumulative event free survival estimates according to P-gp expression in leukemic blasts at diagnosis ($N = 99$; Log-rank test $P = 0.005$).

ALL or ANLL) children with leukemic relapse to be higher ($P = 0.01$) than that in 19 patients at initial diagnosis. Unfortunately in the latter study the samples were unpaired.

Multivariate analysis of the data also reveals that the newly recognized marker of an adverse prognosis is independent of other known risk factors. This inverse correlation of P-gp expression with disease-free survival was also observed by Sauerbrey *et al.* [7]. In this study however, P-gp was not independent from initial peripheral cell count.

Paired sample analysis in 20 patients showed absent P-gp expression in 12 of 14 with relapsed disease, who were P-gp negative at initial diagnosis despite repetitive exposure to chemotherapeutic drugs including anthracyclines and vinca alkaloids. Whether a lack of sensitivity of the applied immunocytochemical method may explain this low score remains to be established. Alternatively, this observation must also mean that other unknown mechanisms procuring drug resistance to cells must exist as is shown by the finding of additional upregulation of multidrug resistance associated protein (MRP) in relapsed childhood leukemia [18]. A possible role of lung resistance protein (LRP) in multiple relapsed ALL samples support such view and support the hypothesis of the multifactorial nature of cellular drug resistance [12]. It has been observed that P-gp positive relapsed ALL patients have a lower probability of a second complete

TABLE II Overall survival in relapsed group according to different prognostic variables. RR_u: Univariate risk ratio (95% confidence interval. RR_m: Multivariately adjusted risk ratio (95% confidence interval) according to Cox proportional hazard modeling

Prognostic factors	% Dead of leukemia	Mortality rate per 1000 person years	RR_u	P	RR_m	P
Age						
1–10 years	37.0 (10/27)	98.6	1		1	
<1 or >10 years	70.0 (7/10)	651.2	4.72 (1.60–13.89)	0.005	2.36 (0.72–7.81)	0.16
Sex						
Female	58.8 (10/17)	300.0	1		1	
Male	35.0 (7/20)	88.8	0.37 (0.14–1.01)	0.06	0.31 (0.10–0.96)	0.04
Time relapse/diagnosis						
≥30 mo	30.0 (6/20)	67.8	1		1	
<30 mo	64.7 (11/17)	464.8	6.36 (2.24–18.11)	<0.001	4.92 (1.36–17.84)	0.02
P-gp expression at relapse						
Negative	33.3 (8/24)	104.3	1		1	
Positive	69.2 (9/13)	253.5	2.18 (0.84–5.67)	0.11	1.46 (0.53–4.03)	0.46

remission or a worse overall survival compared with the P-gp negative relapsed patients [6,17]. In this study, P-gp positive patients at relapse have a two-fold higher risk for leukemic fatal outcome. This relative risk even could have been higher since an allogeneic bone marrow transplantation was successful in two P-gp positive patients with relapsed ALL. Possibly, transplantation might have been a way of overcoming the P-gp mediated multiple drug resistance in those patients.

The data presented and the conclusion drawn should stimulate the clinician to look for multidrug resistance on the leukemic cells of every newly diagnosed child with ALL. In addition, this underscores the usefulness of research work on the modulation of multidrug resistance in tumor cells.

Acknowledgements

The excellent technical support of Miss Jeannine Geerinck is gratefully acknowledged. We also thank the technicians of the laboratory of the Department of Medical Genetic (Ghent University Hospital) for supply of the K562 and K562/VLB cell lines. This work was partially supported by an F.W.O. "Aspirant Kom Op" grant to Barbara De Moerloose.

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THE COMBINED ANALYSIS OF P-GLYCOPROTEIN EXPRESSION AND ACTIVITY PREDICTS OUTCOME IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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The combined analysis of P-glycoprotein expression and activity predicts outcome in childhood acute lymphoblastic leukemia

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Abstract
The link between drug resistance and relapse has often been suggested, but rarely demonstrated in long-range clinical studies. Previously, we used immunocytochemistry to detect the multidrug resistance protein P-glycoprotein (P-gp) in childhood acute lymphoblastic leukaemia (ALL) and we clearly demonstrated the potential of this technique to predict relapse. Since it is nowadays recommended to validate immunocytochemical results by PCR or functional assays, we combined, in the present prospective study, the immunocytochemical APAAP assay with a functional flow cytometric test using Rhodamine 123 and verapamil. During the median follow-up time of 40 months, 10 of 52 included children relapsed. The event-free survival (EFS) and overall survival (OS) at 4 years were 79.3% and 85.2%, respectively. Patients scoring positive in both the immunocytochemical test and the functional assay had a significantly higher relapse rate and worse survival (Logrank P=0.007 and 0.047 for EFS and OS, respectively). Among the different prognostic variables evaluated in this study, only the combination of P-gp expression and activity was a statistically significant parameter predicting relapse in childhood ALL.
In the present study, the combination of immunocytochemistry and a functional detection technique accurately identifies ALL patients with increased risk for relapse.

Keywords: P-glycoprotein, multidrug resistance, childhood acute lymphoblastic leukemia, immunocytochemistry, flow cytometry, outcome

Introduction
Acute childhood leukemias account for 25 to 30% of all childhood malignancies. The predominant type of leukaemia in childhood is the acute lymphoblastic leukemia (ALL) [1]. After chemotherapy was introduced, it was clear that childhood ALL could be divided into many prognostically distinct subtypes and the concept of risk-adapted therapy was introduced [2,3]. In childhood ALL, the following prognostic factors are frequently used: white blood cell count (WBC) at diagnosis, response to treatment, age of the patient at diagnosis, immunophenotype of the lymphoblasts, chromosomal aberrations and minimal residual disease [1,3-5]. Despite risk-adapted therapy, relapse occurs in 25% of the patients.
In a previous prospective study, we demonstrated the prognostic significance of the multidrug resistance (MDR) protein P-glycoprotein (P-gp) in childhood ALL [6]. P-gp is a 170 kD transmembrane protein, capable of extruding a variety of lipophilic compounds, including amphipathic neoplastic agents, out of the cell. Consequently, the intracellular drug concentration decreases and the cell becomes resistant to the drug [7]. Different structurally and functionally unrelated chemotherapeutic agents frequently used in the treatment of ALL, such as anthracyclines, vinca alkaloids and epipodophyllotoxins, are substrates of P-gp action.
P-gp detection in patient samples can be performed by various techniques, which limits the comparison of the results of different clinical studies. Therefore, different P-gp detection assays

have been evaluated in several workshops [8,9]. At the moment, it is recommended to validate immunocytochemical test results by complementary tests such as functional flow cytometric assays or reverse transcriptase-polymerase chain reaction tests.
In our previous study, an immunocytochemical assay was used [6]. In the present prospective study in childhood ALL, we combined immunocytochemical test results with P-gp flow cytometric activities, in order to determine which test or test combination gives the best prediction of relapse.

Patients and methods

Patient characteristics

From June 1996 to January 2000, 61 newly diagnosed ALL patients were admitted to the Department of Paediatric Hematology/Oncology of the Ghent University Hospital.
Out of these, 52 patients were included in the study and their prognostic variables are listed in Table 1. Nine children were excluded from the study. No material was available for P-gp determinations in 8 patients and one child died because of toxicity during induction chemotherapy.

Table 1 Distribution of prognostic factors

Prognostic factor:		Number of patients (%)
Age	1-10 years	38 (73 %)
	< 1 or > 10 years	14 (27 %)
WBC count	$< 50x10^9/l$	39 (75 %)
	$> 50x10^9/l$	13 (25 %)
Cytogenetic analysis	Hyperdiploidy	1 (2 %)
	Unfavourable	5 (10 %)
	Other	44 (85 %)
	Not determined	2 (4 %)
Blasts at day 8	$< 1x10^9/l$	47 (90 %)
	$> 1x10^9/l$	5 (10 %)
DNA content	> 1.16 and < 1.6	5 (10 %)
	< 1.16 and > 1.6	46 (88 %)
	Not determined	1 (2 %)
Gender	Female	28 (54 %)
	Male	24 (46 %)
Immunophenotype	B-cell lineage	44 (85 %)
	T-cell lineage or biphenotypic	8 (15 %)

Hyperdiploidy was defined as > 50 chromosomes in the blasts.
Unfavourable cytogenetic analysis included t(9;22), t(4;11), 11q23 and near-haploidy

The clinical and biological characteristics and the relapse rate of these excluded patients were not significantly different from the studied population. The diagnosis was based on standard morphology, cytochemistry and immunophenotyping of the leukaemic blasts. All patients were treated according to EORTC-CLCG (European Organization for Research and Treatment of Cancer - Childhood Leukemia Cooperative Group) protocols (45 with protocol 58881 and 7 with protocol 58951), including vincristine and anthracyclines. Six children, including the two patients with t(9;22), underwent a bone marrow (BM) transplant. The mean follow-up time was 39 months (median 40 months).

Leukemic samples

Leukemic cells were collected from BM and/or peripheral blood.
For immunocytochemistry, smears of BM and/or peripheral blood were air-dried for 24 hours at room temperature and stored unfixed at -20°C.
For flow cytometry, mononuclear cells from BM and/or peripheral blood were isolated by gradient centrifugation on Ficoll-hypaque, kept at 4°C and analysed within 24 hours after prelevation. All samples contained more than 80% leukemic cells, based on morphological and immunological criteria. Dead cells were excluded with propidium iodide staining.

Control cell lines

Samples of the well-characterised human myeloid leukemia cell line K562 and its P-gp expressing resistant clone K562/VLB20 [10] were included in each immunocytochemical and flow cytometric experiment as negative and positive controls, respectively.

Monoclonal antibodies
In the immunocytochemical assay, P-gp was detected by two monoclonal antibodies (MoAbs) which recognise different extracellular epitopes of P-gp, namely 4E3 (Dako Corporation, Carpinteria, USA) and MRK16 (Kamiya Biomedical Company, Thousand Oaks, USA). The optimal concentration for 4E3 and MRK16 was found to be 10 µg/ml and 5 µg/ml, respectively.

Immunocytochemical technique
P-gp expression on the leukaemic blasts was demonstrated using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) immunocytochemical technique as described previously [11]. Among the different fixatives tested, 0.0125% glutaraldehyde in phosphate-buffered saline (PBS, pH 7.2) was found to be the optimal fixative to preserve both antigen expression and cellular morphology. The slides were fixed for 30 seconds at 4°C, washed and incubated with the MoAb in a humidifier at room temperature for 30 min. The APAAP (APAAP-Dakopatts, Glostrupp, Denmark) staining procedure was performed as prescribed by the manufacturer [12]. Higher assay sensitivity was achieved by repeat incubations with the rabbit anti-mouse antibodies and APAAP complex for an additional 5 minutes. Subsequently, the slides were developed with Fast-Red TR Salt (Sigma, St Louis, USA) and counterstained with gallocyanin. Isotypic matched controls and controls without primary antibody were simultaneously examined to exclude atypical or background staining. Slides were examined by three independent observers. In agreement with the consensus recommendations on P-gp detection, no arbitrary minimal cut-off points were used [8]. In each experiment, a mean of 300 blasts were counted. In slides with poor cellularity, at least 50 blasts were counted. A patient was defined positive for P-gp expression if a red staining pattern along the cell membrane was observed with one of the MoAbs in at least one blast.

Flow cytometric detection of P-gp activity
According to Ludescher et al. [13], 10^5 cells were incubated for 1 hour at 37°C with 200 ng/ml rhodamine 123 (Rho 123, Sigma, St. Louis, USA) which is a P-gp substrate, in the absence or presence of 10 µM of the P-gp inhibitor verapamil (Knoll AG, Ludwigshafen, Germany). The intracellular Rho 123 accumulation was measured on a FACSort flow cytometer (Becton Dickinson, Erembodegem, Belgium) calibrated with QC Windows and Quantum 1000 beads (Flow Cytometry Standards Corporation, San Juan, USA). At least 10.000 events were counted and analysed with Cell Quest software (Becton Dickinson, Erembodegem, Belgium). Cells were washed twice with cold (4°C) HBSS without phenol red and resuspended. After an additional incubation for 1 hour at 37°C with or without 10 µM verapamil, the remaining intracellular Rho 123 retention after the efflux period was measured.
A test result was considered positive if the cells exhibited Rho 123 efflux and if the intracellular Rho 123 fluorescence enhanced in the presence of verapamil by at least 10%, resulting in a Rho 123 retention ratio (RR) of at least 1.10 [14,15].

Statistical analysis
A comparison of frequency distribution of the different prognostic variables according to the P-gp status was determined by Fisher exact tests. Cumulative event-free survival (EFS) and overall survival (OS) were estimated according the Kaplan-Meier method. Relative impact of baseline variables on event (= relapse or death) was expressed as risk ratios (RR). Univariate analysis was performed according to the Cox proportional hazard modelling. P-values less than 0.05 were considered to be statistically significant.

Results
Remission status, EFS and OS
After induction treatment, complete remission (CR) was achieved in all patients. During the study period, 10 patients relapsed and out of these, 6 children died. Three children suffered from progressive leukaemia; the other three died because of toxicity after BM transplant. The EFS at 4 years was 79.3% (95% confidence interval: 67.4% - 93.4%) and the OS 85.2% (95% confidence interval: 74.4% - 97.6%).

Results of the P-gp detection assays
Using the immunocytochemical technique, 24/52 patients (46%) had a positive test result. The EFS and OS were not significantly different in the P-gp negative group, compared to the P-gp positive group (Logrank P = 0.612 and 0.228 for EFS and OS, respectively).

P-gp activity was found in 12 patients (23%) and was not significantly associated with a higher relapse rate (Logrank P = 0.134) or an increased risk to die (Logrank P = 0.107).
Patients who scored positive in either one of the tests (30/52) did not fare worse than the patients with two negative test results (Logrank P = 0.769 and 0.168 for EFS and OS, respectively). On the contrary,

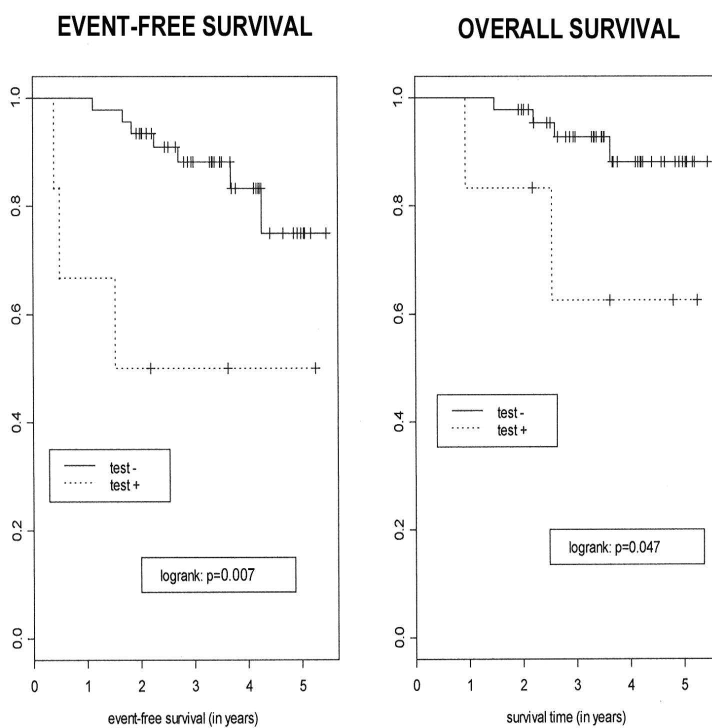
Table 2 Frequency distribution of prognostic variables according to P-gp status

Prognostic factor:		A neg	A pos	P	B neg	B pos	P	C neg	C pos	P	D neg	D pos	P
Age	1-10 years	19	19		30	8		15	23		34	4	
	< 1 or > 10 years	9	5	0.532	10	4	0.713	7	7	0.540	12	2	0.655
WBC count	< $50x10^9$/l	21	18		28	11		16	23		33	6	
	> $50x10^9$/l	7	6	1	12	1	0.253	6	7	0.757	13	0	0.317
Cytogenetic analysis	Hyperdipoid/other	24	21		36	9		19	26		41	4	
	Unfavourable	2	3	0.661	3	2	0.301	2	3	1	3	2	0.103
Blasts at day 8	< $1x10^9$/l	25	22		36	11		19	28		42	5	
	> $1x10^9$/l	3	2	1	4	1	1	3	2	0.639	4	1	0.473
DNA content	> 1.16 and < 1.6	3	2		3	2		2	3		4	1	
	< 1.16 and > 1.6	25	21	1	36	10	0.580	20	26	1	41	5	0.480
Gender	Female	16	12		25	3		15	13		26	2	
	Male	12	12	0.781	15	9	0.045	7	17	0.096	20	4	0.397
Immunophenotype	B-cell lineage	25	19		35	9		20	24		40	4	
	T-cell lineage or Biphenotypic	3	5	0.447	5	3	0.367	2	6	0.442	6	2	0.227

A: Result of the immunocytochemical assay (P-gp positive if either one of the MoAbs is positive)
B: Result of the functional assay (P-gp positive if retention index > 1.10)
C: Combination of both assays (P-gp positive if either the immunocytochemical assay or the functional assay is positive)
D: Combination of both assays (P-gp positive if both assays are positive

the six children with P-gp expression in the immunocytochemical assay and simultaneously a positive functional test had a significantly worse outcome. Three of these six patients relapsed, compared to seven (15%) in thenegative group. Two positive children died, compared to 4/46 (9%) negative children. The cumulative EFS and OS of these patients are shown in Figure 1 (Logrank P = 0.007 and 0.047, respectively).

Figure 1 *Kaplan-Meier event-free survival and overall survival of 52 children with ALL, according to P-gp status (P-gp positive if both assays are positive).*



Evaluation of prognostic factors

All prognostic variables were independently distributed in the P-gp negative and positive patient groups (Table 2). In the patient group scoring positive for the functional assay (test B), a borderline significant dependence (p = 0.045) with gender was seen.

In our study, only the combination of P-gp expression and activity was a statistically significant parameter predicting relapse in childhood ALL (p = 0.021, Table 3). Moreover, the risk to die was 4.49 fold higher in the patient group with a positive result for both P-gp expression and activity, compared to the negative group (p = 0.084, Table 3).

Discussion

The link between drug resistance and relapse has often been suggested, but not defined in clinical treatment settings. The biological markers of drug resistance, such as P-gp, require clinical testing in order to evaluate them as potential therapeutic target.

In the present prospective study, we report the results of immunocytochemical and functional detection of the MDR protein P-gp in childhood ALL and their clinical relevance. Previously, we only used immunocytochemistry for P-gp detection and we clearly demonstrated the potential of this technique to predict relapse [6].

Despite numerous advantages of immuno-cytochemistry (such as discrimination of malignant and normal cells, the evaluation of P-gp staining in individual cells, etc.) [16,17], the French workgroup on P-gp detection does not recommend immunocytochemistry, merely because of a lack in sensitivity and reproducibility [9]. Others promote flow cytometry as the most valuable tool presently available for the detection of MDR in acute leukaemia [18]. The functional flow cytometric assays are, in contrast to immunological tests, independent of the use of MoAbs, and they can directly evaluate the effect of MDR modulators [19]. The sensitivity of the functional test using Rho 123 has been demonstrated in clinical samples [20].

Using the functional test, 23% of our patients scored positive at initial diagnosis. This is in agreement with Tafuri et al. who observed 22.4% positive functional test results in childhood ALL [21]. The immunocytochemical P-gp expression rates in the present study were higher than the P-gp activity values (46% and 23%, respectively). In our previous study [6], only 14% of ALL patients scored positive for P-gp at initial diagnosis We also could not confirm the prognostic value of immunocytochemistry as single test in the present study.

Table 3 Event-free survival and overall survival for ALL patients according to different prognostic parameters

Prognostic factor:		Number of relapses/ Number of patients	RR		P	Number of deaths/ Number of patients	RR		P
Age	1-10 years	6/38	1			3/38	1		
	< 1 or > 10 years	4/14	1.83		0.35	3/14	2.83		0.2
WBC count	< $50x10^9$/l	9/39	1			5/39	1		
	> $50x10^9$/l	1/13	0.299		0.25	1/13	0.651		0.7
Cytogenetic analysis	Hyperdipoid/other	8/45	1			5/45	1		
	Unfavourable	2/5	2.97		0.17	1/5	1.94		0.55
Blasts at day 8	< $1x10^9$/l	9/47	1			5/47	1		
	> $1x10^9$/l	1/5	1.19		0.87	1/5	1.89		0.56
DNA content	> 1.16 and < 1.6	0/5	1			0/5	1		
	< 1.16 and > 1.6	10/46	uncal		uncal	6/46	uncal		uncal
Gender	Female	7/28	1			4/28	1		
	Male	3/24	0.466		0.27	2/24	0.532		0.47
Immunophenotype	B-cell lineage	9/44	1			6/44	1		
	T-cell lineage or Biphenotypic	1/8	0.688		0.72	0/8	0.001		0.83
Test A	negative	5/28	1			2/28	1		
	positive	5/24	1.22	(0.351-4.25)	0.75	4/24	2.48	(0.453-13.5)	0.3
Test B	negative	6/40	1			3/40	1		
	positive	4/12	2.88	(0.808-10.3)	0.10	3/12	3.76	(0.757-18.7)	0.11
Test C	negative	4/22	1			1/22	1		
	positive	6/30	1.18	(0.332-4.22)	0.79	5/30	3.99	(0.465-34.2)	0.21
Test D	negative	7/46	1			4/46	1		
	positive	3/6	4.98	(1.27-19.5)	0.021	2/6	4.49	(0.819-24.6)	0.084

RR: risk ratio (95 % confidence interval); uncal: uncalculable
A: Result of the immunocytochemical assay (P-gp positive if either one of the MoAbs is positive)
B: Result of the functional assay (P-gp positive if retention index > 1.10)
C: Combination of both assays (P-gp positive if either the immunocytochemical assay or the functional assay is positive)
D: Combination of both assays (P-gp positive if both assays are positive)

The discrepancies between the two studies might be caused by the use of different MoAbs: C219 and 4E3 in the former and MRK16 and 4E3 in the present study. MRK16 and 4E3 were chosen because the externally binding MoAbs are more sensitive in the detection of low and variable levels of P-gp and because C219 is not specific for the MDR1 gene product since it also reacts with the MDR3 gene product [8,22].
Current reports on P-gp expression, functional activity and outcome in childhood ALL are conflicting. Goasguen et al. used C219 and JSB1 in an immunohistochemical assay and demonstrated a higher relapse rate and a shorter median survival for P-gp positive patients [23]. Sauerbrey et al. also used C219 and immunocytochemistry and found a lower EFS in the P-gp positive group [24].
In other studies in childhood ALL, flow cytometric functional or immunological assays were performed [15,25]. Kanerva et al. also performed a prospective study in childhood ALL [25]. They used the MoAb JSB1 which recognises an internal epitope of P-gp in an immunological flow cytometric test and found no difference in EFS or OS between children with low or increased P-gp expression. Other researchers prefer the functional flow cytometric assay [15,21]. P-gp activity was studied by Wuchter et al. in 102 children with ALL and did not correlate with immunophenotypic subgroups, response to induction chemotherapy, relapse rate and OS [15].
These conflicting data resulting from prospective studies based on one detection technique, stress the importance of combining complementary tests such as immunocytochemistry and functional assays. Recently, Damiani et al. found a shorter disease-free survival in adults with ALL, if P-gp overexpression was associated with a defect in daunorubicin accumulation [26].
Among the different variables studied in our 52 ALL patients, only the simultaneous positivity of P-gp activity and P-gp expression was significantly associated with a higher risk for relapse. No single test results and no other prognostic parameters in childhood ALL (age, gender, WBC count at diagnosis, number of blasts at day 8, immunophenotype of the blasts, DNA content and cytogenetic abnormalities) had a comparable increased risk for relapse or death.

Conclusion

The combined use of a functional flow cytometric assay with Rho 123 and an immunocytochemical staining of P-gp does not only allow to determine accurately P-gp in clinical samples, as was suggested by others [27], but additionally leads to the identification of patients with increased risk for relapse.

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3 Discussie

In dit hoofdstuk werden de immuuncytochemische test en de flowcytometrische functionele test toegepast op stalen van kinderen met ALL. Er werd nagekeken of P-gp expressie en/of functie een prognostische parameter was voor recidief of overlijden. Bovendien werd de onafhankelijkheid van deze prognostische waarde t.o.v. gekende prognostische factoren nagegaan.

In de eerste studie (Dhooge et al., 2002), een prospectieve studie met een gemiddelde follow-up van 65 maanden, werden 102 nieuw-gediagnosticeerde ALL patiëntjes en 37 gerecidiveerde kinderen immuuncytochemisch onderzocht naar P-gp expressie. Twintig kinderen werden sequentieel getest. In deze studie werden de MoAbs C219 en 4E3 gebruikt. P-gp expressie werd slechts bij 14 % van de stalen bij diagnose teruggevonden. Toch bleek dat de EFS van de P-gp positieve patiënten significant slechter was dan deze van de P-gp negatieve kinderen (Logrank P = 0.005). Bovendien was dit resultaat onafhankelijk van andere prognostische parameters zoals leeftijd, leucocytose bij diagnose en karyotype. Bij recidief waren meer stalen positief dan bij diagnose (35 % versus 14 %). De overleving van deze P-gp positieve kinderen was echter niet significant verschillend van deze van de P-gp negatieve kinderen. Bovendien bleek dat slechts 2/14 initieel negatieve kinderen bij recidief P-gp positief werden.

In de tweede studie (De Moerloose et al., *submitted*) werden 52 kinderen met de novo ALL prospectief onderzocht met immuuncytochemische en flowcytometrische functionele testen, en gemiddeld 39 maand opgevolgd. In deze studie werd nagegaan via welke detectiemethode of combinatie van detectiemethodes de sterkste prognostische waarde van P-gp in ALL op kinderleeftijd teruggevonden wordt. Zoals reeds geopperd werd in hoofdstuk 2 (Swerts et al., *submitted*) geeft de combinatie van de immuuncytochemische APAAP techniek met de flowcytometrische functionele test de meeste prognostische informatie. Immers, enkel de kinderen die positief scoren voor zowel de immuuncytochemische test als de functionele test hebben een significant grotere kans op recidief (Logrank P= 0.007) en op slechtere overleving (Logrank P= 0.047). De patiënten die enkel positief zijn voor de immuuncytochemische test doen het in deze laatste studie niet significant slechter dan de negatieve patiënten.

De discrepantie met het resultaat van onze eerste studie (Dhooge et al., 2002) kan misschien verklaard worden door het gebruik van een ander MoAb. Het antilichaam C219 werd immers vervangen door MRK16 omdat C219 niet specifiek is voor het MDR1 genproduct en omdat het niet gebruikt kan worden in flowcytometrische testen. MRK16, dat net zoals 4E3 een extern epitoop herkent, kan wel toegepast worden in beide immunologische testen.

In de literatuur werd enkel door Goasguen et al. (1993) en Sauerbrey et al. (1994) een prognostische waarde van immuuncytochemische P-gp expressie bij ALL op kinderleeftijd teruggevonden. Opmerkelijk is het feit dat in deze studies ook C219 als MoAb gebruikt werd.
Andere auteurs (Den Boer et al., 1998, Kanerva et al., 2001, Wuchter et al., 2000) onderzochten de prognostische relevantie van P-gp in ALL op kinderleeftijd met immunologische testen of functionele testen en vonden geen significanties terug.

Vermoedelijk zijn deze tegenstrijdigheden het gevolg van het gebruik van niet-gestandaardiseerde technieken en kan enkel een gecombineerde bepaling van P-gp door middel van complementaire testen relevante informatie bieden. Dit werd recent ook teruggevonden in een studie van volwassenen met ALL (Damiani et al., 2002). In deze studie werd een kortere EFS teruggevonden bij patiënten met een P-gp overexpressie en terzelfdertijd een verlaagde daunorubicine-accumulatie, wat een indirecte aanwijzing is van een actieve effluxpomp.

HOOFDSTUK 4

P-GLYCOPROTEÏNE EXPRESSIE EN FUNCTIE BIJ NEUROBLASTOMEN OP KINDERLEEFTIJD

1 Expression of the *MDR1* gene product P-glycoprotein in childhood neuroblastoma

C. Dhooge, B. De Moerloose, Y. Benoit, N. Van Roy, J. Philippé, G. Laureys

Cancer 1997; 80: 1250-1257

2 Discrepant flow cytometric expression and function of P-glycoprotein in neuroblastic tumors

B. De Moerloose, C. Dhooge, G. Laureys, Y. Benoit, M. Demarche, C. Devalck, D. Plantaz, J. Leroy, J. Philippé

Cytometry 1999; 37: 125-132

3 Tc-99m Sestamibi imaging in pediatric neuroblastoma and ganglioneuroma and its relation to P-glycoprotein

B. De Moerloose, C. Van de Wiele, C. Dhooge, J. Philippé, F. Speleman, Y. Benoit, G. Laureys, R.A. Dierckx

European Journal of Nuclear Medicine 1999; 26: 396-403

4 Imaging with technetium-99m sestamibi does not predict drug resistance or outcome in childhood neuroblastic tumours

B. De Moerloose, C. Van de Wiele, G. Laureys, C. Dhooge, K. Swerts, Y. Benoit, J. Philippé, R.A. Dierckx

European Journal of Nuclear Medicine, *submitted*

5 **Discussie**

EXPRESSION OF THE *MDR1* GENE PRODUCT P-GLYCOPROTEIN IN CHILDHOOD NEUROBLASTOMA

C. Dhooge, B. De Moerloose, Y. Benoit, N. Van Roy, J. Philippé, G. Laureys

Cancer 1997; 80: 1250-1257

Expression of the MDR1 Gene Product P-Glycoprotein in Childhood Neuroblastoma

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BACKGROUND. In cancer treated with chemotherapy, multidrug resistance is characterized by increased genetic expression of P-glycoprotein (P-gp), which acts as an ATP-dependent drug-efflux pump. However, the clinical significance of the expression of the multidrug resistance gene (MDR1) product P-gp in neuroblastoma (NB) is still a matter of debate. In this study, the role of the expression of P-gp in NB was evaluated.

METHODS. NB tumor imprints and NB positive bone marrow smears from 23 children before and after multidrug chemotherapy were examined for P-gp expression by antialkaline phosphatase immunocytochemical analysis.

RESULTS. Before chemotherapy, only 10% of the NB samples showed positivity for P-gp. At diagnosis, no difference in P-gp expression was found between primary tumor cells and NB cells from metastases to bone marrow. P-gp positivity was only observed in patients with nonlocalized disease. P-gp positivity was never found in tumor cells that were histologically well differentiated. No clear correlation of P-gp positivity with poor prognostic parameters, such as chromosome 1p deletion or MYCN amplification, were found. Multidrug chemotherapy did not induce enhanced expression of P-gp in the neuroblasts. However, at clinical recurrence, P-gp expression was found in the metastatic NB cells of five of seven bone marrow samples examined.

CONCLUSIONS. The prognostic relevance of P-gp expression in NB was not clear from the results of this study. To resolve the uncertainties, a standardization of the methodology and more prospective studies are needed to determine whether routine analysis of P-gp is worth adding to the other prognostic parameters that are evaluated in NB patients. The finding that metastatic cells are capable of expressing MDR1, in contrast to the NB cells of the primary tumor, would certainly be an interesting topic for further study as work directed at understanding the progression to metastasis continues. ***Cancer* 1997;80:1250–7.**
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KEYWORDS: neuroblastoma, multidrug resistance, MDR1, P-glycoprotein, P-gp, prognostic factors, metastasis.

The discovery of a multidrug resistance protein (MDR), P-glycoprotein (P-gp), in 1985 appeared promising in aiding in the understanding of the mechanisms of failure of antimitotic treatment in patients with cancer.[1] The MDR protein P-gp is an energy-dependent drug-efflux pump of 170 kilodaltons, encoded by the multidrug resistance gene (MDR1) on human chromosome 7, band q21.1.[2] It is highly conserved and is expressed on the cell membrane of normal tissues (colon, liver, adrenal glands, kidney, and pancreas[3]), in which it has an excretory and/or transport function of agents toxic to the cells. The finding of (over)expression of P-gp in patients with hematologic malignancies and various tumors[4,5] led to the hypothesis that overex-

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Received April 21, 1997; accepted May 15, 1997.

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pression of P-gp in a cancer cell might render the cell resistantto various chemotherapeutic drugs by enhancing the efflux of the drugs out of the cell. The study of P-gp expression as a parameter of prognostic clinical value was undertaken for various tumors.

For patients with neuroblastoma (NB), a childhood tumor originating in sympathetic neural crest cells, long term prognosis has not significantly improved during the last 10 years for patients with extensive disease, despite multidrug therapy. This is in contrast to the therapeutic progress made for most other pediatric tumors. Although MYCN amplification and deletion of the short arm of chromosome 1 are correlated with poor therapeutic response and short disease free survival, a large percentage of NB patients lack such parameters, which suggests that other biomolecular mechanisms might be involved in the progression of this tumor. The question was raised whether expression of MDR1 is found in NB and if it is related to adverse clinical outcome. In the literature, conflicting results were reported regarding the functional role of P-gp in human NB and regarding correlation of P-gp expression with multidrug resistance and adverse clinical outcome. Chan et al. were among the first to report a relationship between P-gp expression and prediction of success or failure of therapy for patients with nonlocalized NB.[6] However, other authors failed to reproduce these results, and reported P-gp expression in early stage tumors and in those with well differentiated histology.[7]

Recently, another MDR-related gene has been described: the multidrug resistance-associated protein (MRP) gene. Expression of MRP was found to be correlated with amplification and overexpression of the MYCN oncogene. Recent data suggest that MYCN regulates MRP expression, and that the adverse outcome in MYCN-amplified NB might be due to overexpression of MRP.[8]

In this study, tumor imprints and/or bone marrow (BM) smears in the case of metastatic disease were evaluated for P-gp expression. For most patients other parameters of prognostic significance such as age, stage, chromosome 1 status, DNA ploidy, and MYCN amplification were available.

MATERIALS AND METHODS

Patients: Clinical Characteristics

From January 1990 until September 1995, 23 patients age < 16 years with the diagnosis of neural crest tumor who were admitted to the Department of Pediatric Hematology/Oncology at the University Hospital of Gent were evaluated for P-gp expression. Nine patients admitted during the same period were excluded because of the lack of a tumor specimen for evaluation of P-gp expression. Informed consent was obtained from all patients and/or their parents/guardians. Patient characteristics are listed in Table 1.

All patients underwent control examinations every 3 months that included screening for catecholamines and their byproducts, neuron specific enolase, lactic dehydrogenase, ferritin, computed tomography (CT) scans, BM samples, metaiodobenzylguanidine (MIBG) scans, and CT/magnetic resonance imaging scans of the primary tumor sites.

Treatment was dependent on the patient's age, tumor stage (according to the International Neuroblastoma Staging System), and clinical symptoms. All patients with low grade, Stage 1 NB (Cases 7, 17, and 20) and two patients with Stage 4S (Cases 3 and 22) disease underwent surgery alone. Multidrug chemotherapy according to the infant protocol of the European Neuroblastoma Study Group (ENSG NB 9205) was given to one infant with Stage 4S NB (Case 18) because of a rapid enlargement of the liver resulting in breathing difficulties as well as to one infant with Stage 2B NB (Case 19) according to the protocol instructions. Patients with Stage 3 and 4 disease were given combination chemotherapy for approximately 1 year comprised of platinum derivatives, vinca alkaloids, anthracyclines, antimetabolites, alkylating agents, and podophyllotoxins. When no further tumor regression could be obtained and on the condition that no metastatic disease was demonstrable, patients proceeded to surgical resection or gross debulking of the primary tumor. When an MIBG positive mass still was present after surgery, patients received external irradiation and/or radionated MIBG (^{131}-MIBG) therapy (Cases 1, 2, 6, 10, 12, and 13). Patients with gross tumor resection with evidence of immature elements on histologic examination (ganglioneuroblastoma) and no positive MIBG uptake after surgery were treated with maintenance therapy. In three patients consolidation with an autologous BM transplantation was performed (Cases 2, 6, and 11). The patient in Case 15 had an unusual presentation of disease. During infancy, he was treated for Stage 1 NB with surgery alone. At the age of 2.5 years he developed BM metastasis and was treated with multichemotherapy for 1 year. At last follow-up, the patient had been disease free for >20 months.

Progression free survival (PFS) time and overall survival are expressed in months from diagnosis.

For most tumor samples, evaluation of the following parameters was performed: chromosome 1p deletion, MYCN amplification, and DNA index (the latter in the most recent samples). Besides classic karyotyping to assess copy number of chromosome 1 and presence of chromosome 1p deletions, dual-color fluorescent in situ hybridization (FISH) was performed on disaggregated fresh tumor cells. The following probes

TABLE 1
Characteristics of 23 Children with Neuroblastoma

Patient	Age at diag. (mos)	Stage	Del chromosome 1p	MYCN ampl	DNA index	P-gp C219/ 4E3	Outcome
1	112	4	T: disomic	0	0	BM: neg/neg T: neg/neg T*: 0/neg	Dead (26 mos)
2	32	4	T*: disomic	0	0	BM: neg/neg	Dead (38 mos)
3	1	4S	0	0	0	T: neg/neg	PFS (104 mos)
4	20	4	T: del of 1p	T: pos	1.17	T: neg/0	Dead (6 mos)
5	47	4	T: disomic	0	0	BM: neg/0 T: neg/0	Dead (36 mos)
6	84	4	T*: disomic	T*: neg	0	T: neg/pos BM**: neg/pos	Dead (40 mos)
7	16	1	T: disomic	T: neg	1.43	T: neg/neg	PFS (53 mos)
8	54	4	T: disomic	T: neg	1.09	T: neg/neg	PFS (16 mos)
9	18	4	BM: del 1p	BM: pos	0	BM**: neg/pos	Dead (13 mos)
10	56	4	T: disomic	T: neg	1	T: neg/neg BM**: 0/pos	PD (41 mos)
11	20	3	T: del 1p	T: neg	0	T: neg/0	Dead (22 mos)
12	66	4	T: disomic	T: neg	0	T: neg/neg	PD (39 mos)
13	34	4	T: disomic	T: pos	0	BM: 0/neg T: neg/neg BM**: pos/pos	Dead (28 mos)
14	7	2	T: trisomic	T: neg	1.34	T: neg/neg	PFS (40 mos)
15	30	4	BM: trisomic	BM: neg	1	BM: neg/neg	PFS (30 mos)
16	36	4	BM: disomic T*: disomic	BM: neg T: neg	1.09	BM: pos/pos T*: neg/neg	PFS (25 mos)
17	22	1	T: trisomic	0	1.53	T: neg/neg	PFS (22 mos)
18	4	4S	T: trisomic T*: trisomic	T: neg T*: neg	1.31	T: 0/0 T*: neg/0	PFS (25 mos)
19	10	2	T: trisomic	T: neg	1.28	T: neg/neg	PFS (25 mos)
20	142	1	T: disomic	T: neg	1	T: neg/neg	PFS (14 mos)
21	2	4S	T: trisomic	T: neg	1.74	T: neg/pos	PFS (43 mos)
22	50	4	T: disomic	T: neg	0	T: neg/neg	PFS (8 mos)
23	57	4	T: del 1p	T: pos	1.13	T: neg/neg	PD (13 mos)

Diag: diagnosis; Del: deletion; ampl: amplification; P-gp: P-glycoprotein; nd: not done; BM: bone marrow; BM**: bone marrow at recurrence; T: tumor; T*: tumor examined after multidrug chemotherapy; 0: no material evaluable for examination; PD: progressive disease; PFS: progression free survival; MYCN amplification: positive if >6 copies; neg: negative; pos: positive.

were used: pUC1.77 (heterochromatic probe for chromosome 1)[9] and p1-79 (subtelomeric probe for chromosome 1).[10] For the determination of MYCN amplification (MYCN copy number > 6), FISH of nuclei from tumor cells and Southern blot analysis of tumor DNA were performed with the MYCN probe (pNb-9) (a gift from Dr. M. Schwab) or pNb-1, respectively. FISH was performed as previously described.[11] The DNA index was defined as the ratio of the modal G_0/G_1 peak channel number for NB cells to that of normal cells performed on an EPICS PROFILE II flow cytometer (Coulter Cytometry, Hialeah, FL).

Evaluation of MDR1 Gene Product

Sample collection

P-gp on human NB cells was examined using an alkaline phosphatase-antialkaline phosphatase immunocytochemical analysis (APAAP). Tumor imprints obtained before chemotherapy and BM aspirates in case of metastatic involvement were air-dried for 24 hours and then frozen at −20 °C to increase permeability of the cell membrane. Immunocytochemistry was performed as described[12] with the primary monoclonal antibody (MoAb) C219 (Centocor) and the MoAb 4E3 (Centocor) at a final working concentration of 20 μg/mL and 10 μg/mL, respectively. C219 and 4E3 are both mouse antibodies. C219 recognizes an internal epitope on the MDR1 and MDR3 isoform of the P-gp molecule.[13] The MoAb 4E3 is an affinity-purified antibody that recognizes only the epitope of the MDR1 gene, which is located on the exterior surface of the plasma membrane. 4E3 does not cross-react with the MDR3 gene product.[14] The sensitivity of this technique was improved by adding a tertiary layer of mouse antirabbit antibodies plus APAAP complex after the use of rabbit antimouse antibodies.[12] For each experiment slides without incubation with primary antibody were included as isotypic matched controls.

The feasibility of immunocytochemical assessment of the specific reactivity of the MoAb C219 and the MoAb 4E3 was first demonstrated in the in vitro cultured cell line K-562 and its resistant clone K-562/VLB.[15] The K-562 cell line is a human myelogenous leukemia cell line lacking any reactivity with 4E3 and C219 (negative control). Its multidrug resistant clone K-562/VLB shows a multidrug resistant phenotype with cross-resistance to various drugs, an increased efflux and a significant decrease in steady-state accumulation of these drugs, and an increased expression of P-gp.[15] The latter cells show clearly positive staining with both MoAbs.

Both the resistant clone and the sensitive parental cell line were used as positive and negative controls, respectively.

Using immunocytochemistry with enhanced sensitivity on imprints from fresh tumor tissue and/or on BM smears of samples with clear NB tumor metastasis, the authors looked for P-gp on intact tumor cells, thus avoiding confusion with other cell types. Immunocytochemistry, as used in this experiment, can be used as a semiquantitative method for P-gp evaluation. However, because all the slides were not screened on a quantitative base, only positive and negative scores were used.

The results were interpreted by three independent observers. BM smears and tumor imprints were scored as positive if a red staining pattern along the cell membrane was observed in at least 5% of the blasts (Fig. 1).

Statistical Analysis

The statistical endpoints were progression free survival (PFS) and overall survival. Events were defined as progressive disease or as recurrent disease after achievement of complete remission. The mean follow-up for those patients who experienced an unfavorable outcome was 26 months (time since diagnosis until death). The mean follow-up for the patients at last follow-up was 32.8 months, with a median of 27.5 months. Table 2 lists the distributions of the prognostic factors examined in the patients. Fisher's exact test was used to compare risk groups and the rate ratio (RR) was calculated to quantify the relation between the risk factor under study and events in univariate analysis (Table 3).

RESULTS

Stage, Age, and Outcome

Event free survival for patients with Stage 1 (3 cases), 2 (2 cases) and 4S (3 cases) NB was 100% with a median follow-up of 32.5 months. Amplification of MYCN and/or chromosome 1p deletion were not observed in patients with Stage 1, 2, and 4S NB. DNA ploidy showed triploidy (three chromosomes 1 and/or DNA index > 1.18 and < 1.8) in almost all cases with Stage 1 and 2 disease and Stage 4S disease, except for one older child (Case 20). No data concerning ploidy status were available for one Stage 4S patient. In two patients progression of disease was observed; both patients were rescued with chemotherapy (Cases 18 and 19).

Overall survival in patients with Stage 3 and 4 NB was poor and comparable to that reported by other groups (3-year overall survival for Stage 3 and 4 disease was 50%). Eight of 15 patients experienced an unfavorable outcome, and at last follow-up 3 patients were alive with progression of disease (Table 3). Four patients (Cases 8, 15, 16, and 22) with Stage 4 disease were progression free with a mean follow-up of 20 months (Table 1).

P-gp in Human NB

Tumor specimens were examined for P-gp expression before and after multichemotherapy. Of the 54 samples examined with the APAAP immunocytochemical method, clear positive staining was found in 9.

Four of 43 tumor samples (primary tumor material and/or BM in the case of Stage 4 disease) were positive for P-gp at diagnosis (9.3%); all patients had nonlocalized disease. There was no P-gp expression in patients with low grade NB.

The authors could not find P-gp positivity in the four

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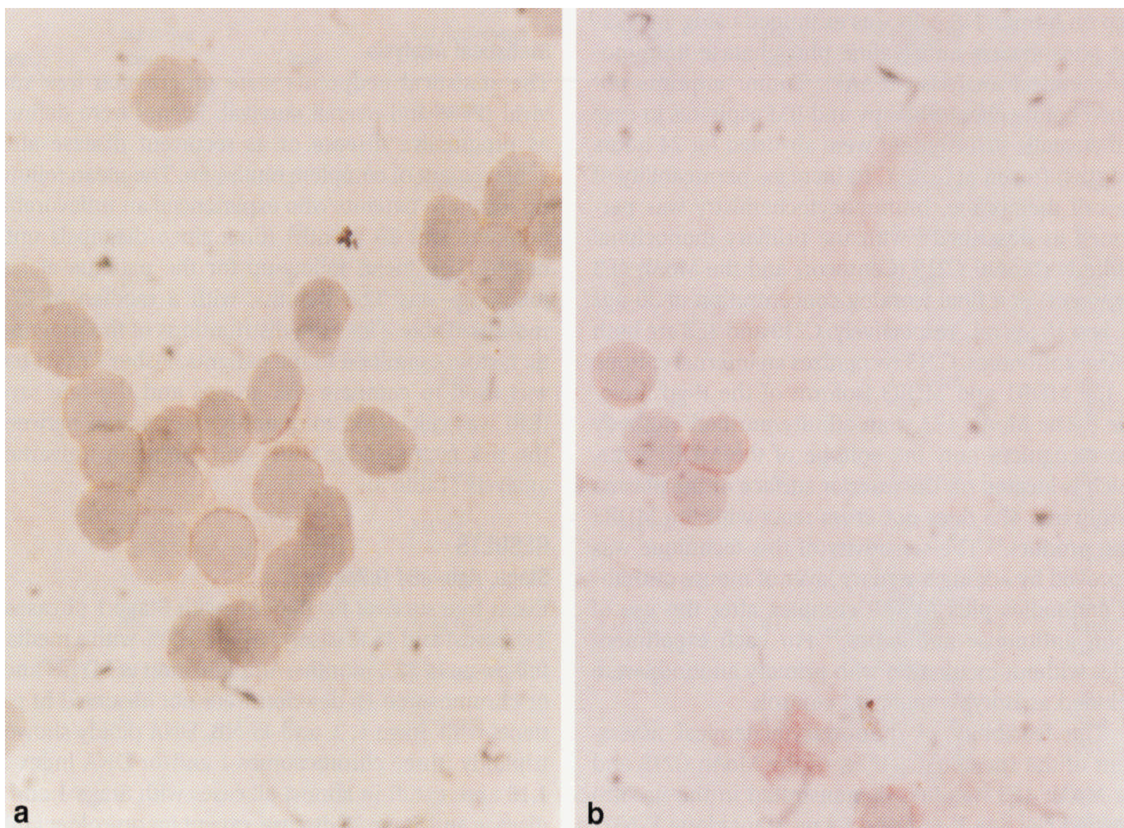

FIGURE 1. (a) Tumor imprint (Case 21) showing a red staining pattern along the cell membrane of the neuroblastoma (NB) cells using the alkaline phosphatase-antialkaline phosphatase immunocytochemical analysis (APAAP) method with the monoclonal antibody (MoAb) 4E3. (b) Bone marrow slide (Case 13) at clinical recurrence, expressing P-glycoprotein on the NB cells (APAAP with the MoAb 219).

TABLE 2
Distribution of Prognostic Factors Examined

	No.	%
Age <12 mos	5	22
>12 mos	18	78
Stage: 1, 2, 4S	8	35
3, 4	15	65
Disomic	12	55
Trisomic	6	27
Del chromosome 1p	4	18
NYCN ampl		
Neg	14	78
Pos	4	22
DI 1.18–1.8	6	46
<1.18 or >1.8	7	54
P-gp samples		
Neg	45	83
Pos	9	17

Del: deletion; ampl: amplification; Neg: negative; Pos: positive; DI: DNA index; P-gp: P-glycoprotein.

samples of material from the primary tumor obtained by surgery after several months of multichemotherapy, all samples in which NB cells were clearly present.

BM samples, containing metastatic disease (Stage 4) obtained at diagnosis were examined in six cases (Cases 1, 2, 5, 13, 15, and 16). All except one (Case 16) were negative for P-gp. However, at recurrence after several months of multichemotherapy, P-gp expression was found in five of seven BM smears containing metastatic disease. The rate ratio for P-gp positivity to predict an event was 0.69, which is not significant compared with the MYCN amplification (RR: 7) and chromosome 1p-deletion (RR: 4) calculated for the same group of patients (Table 3). However, the RR to predict death was 1.64, meaning that a NB patient with P-gp positivity was 60% more at risk to have an adverse clinical outcome compared with a P-gp negative NB patient.

MYCN and Chromosome Abnormalities and Outcome

MYCN amplification was found to be present in 4 of 11 children with Stage 4 disease. Deletions of the short

TABLE 3
Relation between Risk Factors and Events (Univariate Analysis)

	% Event	P^a value	Event rate (/5 yr)	RR
Age < 12mos	0.0% (0/5)		0.0	1
>12 mos	61.1% (11/18)	0.04	1.4	∞
Stage 1, 2, 4S	0.0% (0/8)		0.0	1
3, 4	73.3% (11/15)	0.001	8.75	∞
Disomic	58.3% (7/12)		1.15	1
Trisomic	0.0% (0/6)		0.0	0
Del chromosome				
1p	100% (4/4)	0.004	4.44	3.9
MYCN				
Neg	28.6% (4/14)		0.57	1
Pos	100.0% (4/4)	0.02	4.00	7.0
DI 1.18–1.8	0.0% (0/6)		0.0	1
<1.18 or >1.8	42.9% (3/7)	0.19	1.24	∞
P-gp				
Neg	41.2% (7/17)		0.81	1
Pos	33.3% (1/3)	0.99	0.56	0.69

RR: rate ratio; del: deletion; neg: negative; pos: positive; DI: DNA index; P-gp: P-glycoprotein.
[a] According to Fisher's exact test.

arm of chromosome 1 were found in 4 of 15 patients with Stage 3 or 4 disease. All five patients with one of the markers mentioned earlier died of their disease or were alive with disease progression at last follow-up. Amplification of MYCN and/or chromosome 1p deletions were not observed in the 4 patients with Stage 4 disease who were free of disease progression at last follow-up (Cases 8, 15, 16, and 22) nor in patients with Stage 1 and 2 disease or Stage 4S disease.

Trisomy 1 was found in six patients: three with localized disease, two with Stage 4S, and one patient (Case 15) with Stage 4 disease at recurrence. All were alive with progression free disease at last follow-up. There was a good correlation between the FISH results and the DNA index.

DNA Ploidy and Outcome

DNA content was measured in 13 patients. At last follow-up, 6 of the 6 patients with a DNA index between 1.18–1.8 were alive and all had good prognostic parameters (e.g., Stage 1, 2, and 4S disease, absence of MYCN amplification, and no deletion of chromosome 1p).

Monoclonal Antibodies

Both MoAbs were used on the same tumor imprints whenever there was enough material to examine. Because it was MDR1 specific, 4E3 was slightly more sensitive compared with C219 in this setting. Another advantage of the new MoAb 4E3 is that it recognizes an external epitope of P-gp, so that the permeabilitization of the cell membrane is no longer required.

DISCUSSION

Overexpression of P-gp might render malignant cells resistant to various chemotherapeutic drugs. In the literature, controversies remain as to whether the overexpression of P-gp might be responsible for the adverse clinical outcome in a subset of NB patients with no other adverse prognostic parameters. Conflicting data were even reported regarding the absence or presence of P-gp overexpression in a percentage of neuroblastoma tumors and with regard to its correlation with the degree of differentiation.

In this study, P-gp expression, as evaluated by an APAAP immunocytochemical method, was found in 10% of NB samples at diagnosis. Positivity for P-gp was not uniformly distributed within one tumor sample. In most positive samples, a variable amount of tumor cells (up to a maximum of 30%) stained positive for P-gp, which suggests heterogeneity within a tumor. These results are in agreement with those reported by Bénard et al., who obtained a positivity of 20% for P-gp in NB samples at diagnosis using the same immunocytochemical technique.[16] With Northern blot hybridization P-gp positivity at diagnosis increased to 71%, as described by the same authors.[16] However, when techniques

that work with homogenized samples are applied, such as Northern blot analysis, slotblot analysis, or polymerase chain reaction analysis, overestimation of the multidrug resistant content of tumor cells must be suspected because no discrimination is made between normal and malignant cells as was mentioned several times in the literature.[17,18]

No difference in P-gp expression was found in imprints of the primary tumor at diagnosis compared with solid tumor samples obtained during second surgery after several months of chemotherapy. Histologically, the tumor samples used in the current study still showed NB cells, as well as more differentiated areas to ganglioneuroblastoma and areas with necrosis. This is in contrast to the results of Bénard et al.,[19] who, even with immunocytochemistry, found enhanced expression after chemotherapy.

When treated by retinoic acid, neuroblasts exhibited marked neuronal differentiation and a significant increase of transcript levels of the human MDR1 gene.[20] Ferrandis et al.[21] reported a trigger of human MDR1 gene transcription in an NB cell line in which neuronal differentiation was induced. However, in this study no positive correlation was found between P-gp expression and the degree of maturation of the tumor cells. In the current study, all the P-gp positive samples were found in histologically poorly differentiated tumors from children with nonlocalized disease (including a patient with Stage 4S disease).

The finding of a P-gp positive NB was not found to be a statistically significant parameter to predict progressive disease, nor will it point to an evolution of recurrence in this study (RR 0.69) (Table 3). However, the relative risk of death increased by 60% if the NB patient was P-gp positive. Other well known parameters such as age, stage, MYCN amplification, and deletion of chromosome 1p were statistically significant ($P < 0.05$). The DNA index was not significant, probably because of the number of patients. In this study no adverse biologic prognostic parameters (deletion of chromosome 1p, MYCN amplification) were found in patients with low grade NB. Two patients had only one of these parameters present (Cases 11 and 13); both experienced an adverse clinical outcome. Interestingly, P-gp positivity was found in 5 of 7 NB positive BM samples at recurrence, whereas nearly all (eight of ten) NB positive BM samples were negative for P-gp expression at diagnosis. This observation suggests that metastatic cells have the capacity to express P-gp and to extrude toxic drugs after exposure to chemotherapeutics, in contrast to the NB cells of the primitive tumor. The finding that an increase in MYCN and MDR1 transcript levels was associated with the metastatic process was reported in the literature by Ferrandis et al.[21] in a human NB xenograft model (IGR-N-91) and the same conclusions reached from the current clinical prospective study confirm the previous hypothesis.

Currently, it is too early to encourage routine analysis of P-gp as a clinical screening test for MDR in NB patients to tailor chemotherapeutic and other regimens to individual patient needs. However, the finding that metastatic cells are capable of expressing MDR1 disease in contrast to the NB cells of the primary tumor is certainly an interesting topic for further study toward the understanding of tumor progression to metastasis.

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DISCREPANT FLOW CYTOMETRIC EXPRESSION AND FUNCTION OF P-GLYCOPROTEIN IN NEUROBLASTIC TUMORS

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Cytometry 1999; 37: 125-132

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Cytometry 37:125–132 (1999)

Discrepant Flow Cytometric Expression and Function of P-Glycoprotein in Neuroblastic Tumors

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Received 5 August 1998; Revision Received 11 February 1999; Accepted 29 May 1999

Background: Patients suffering from neuroblastic tumors are currently being classified into prognostic subsets based on different clinical and biologic features. In this study, a triple-color flow cytometric assay and a functional test were applied to neuroblastoma cell lines and patients with a neuroblastic tumor, and the value of P-glycoprotein expression and function as potential prognostic characteristics, was determined.
Methods: Twenty-two single-cell suspensions prepared from tumors, and neuroblasts from four bone marrow samples were analyzed by triple-color flow cytometry. Neuroblasts were identified by NB84-positivity and absence of CD45. P-glycoprotein expression was evaluated using 4E3 and MRK16 antibodies. Eighteen samples were tested with a functional assay, based on accumulation and retention of rhodamine-123 with and without the inhibitor verapamil. Six neuroblastoma cell lines were also evaluated.
Results: P-glycoprotein expression was seen in 18 of 26 patient samples and in three of six cell lines. The highest expression levels were found in low stage neuroblastoma and well-differentiated tumors; whereas the highest activities were found in stage 4 neuroblastoma and the lowest in ganglioneuroblastoma and ganglioneuroma patients. In 10 of 17 samples, concordant results were found between the flow cytometric immunological test and immunocytochemistry.
Conclusions: The described flow cytometric technique is a new, alternative approach to detect P-glycoprotein expression and function in neural crest tumors. Based on the expression level and the activity value, patients can be segregated into different phenotypic groups. In particular, those patients with high P-glycoprotein activity might benefit from treatment regimens containing reversal agents. Cytometry 37:125–132, 1999. © 1999 Wiley-Liss, Inc.

Key terms: P-glycoprotein; multidrug resistance; neuroblastoma; ganglioneuroma; flow cytometry; rhodamine-123; NB84

The neuroblastic tumors, including neuroblastoma (NB), ganglioneuroblastoma (GNB), and ganglioneuroma (GN) originate in sympathetic neural crest cells. They demonstrate diverse biological characteristics and clinical behaviors, from differentiation into benign tumors or even spontaneous regression to aggressive disease. In particular for patients with extensive disease, the long-term prognosis has not significantly improved during the last 10 years despite the introduction of multidrug therapy (7). Although amplification of the MYCN oncogene and deletion of the short arm of chromosome 1 are known to be correlated with poor therapeutic response and short disease-free survival (7,10,39), a large percentage of NB patients lack such markers, which suggests that other biomolecular mechanisms might be involved in the progression of this tumor.

One of the causes of chemotherapy failure is the presence or the development of multidrug resistance (MDR), a cross-resistance to a wide range of structurally and functionally unrelated drugs (22). MDR is frequently associated with amplification or increased expression of the *MDR1* gene and overproduction of its product, P-glycoprotein (P-gp), a 170-kDa plasma membrane protein that was first isolated by Riordan and Ling (34). P-gp is expressed not only in tumors but also in several normal human tissues with secretory or barrier functions, as well

Grant sponsor: F.W.O. Kom op tegen kanker.
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Table 1
Patient Profiles

Patient	Age (mo)	Sex	Diagnosis[a]	Stage (INSS)	Chemotherapy	Response	Outcome (follow-up)
1	27	M	NB	4	yes	no	dead (9mo)
2	35	M	NB	4	yes	yes, relapse	alive(24mo)
3	10	M	NB	4	yes	yes	alive (9mo)
4	15	F	NB	4	yes	yes	alive (4mo)
5	54	F	NB	4	planned		alive (0mo)
6	82	M	NB	4	yes	no	alive(11mo)
7	17	M	NB	3	yes	yes, relapse	dead (7mo)
8	0	F	NB	3	yes	yes, relapse	dead (7mo)
9	18	F	NB	2a	no		alive(14mo)
10	15	F	NB	1	no		alive(21mo)
11	47	F	NB	1	no		alive(11mo)
12	5	M	NB	1	no		alive(10mo)
13	3	M	NB	1	no		alive (8mo)
14	65	M	NB	1	no		alive(13mo)
15	4	F	NB	1	no		alive (1mo)
16	122	M	NB Relapse		yes	yes	alive(10mo)
17	42	F	GNB	1	no		alive (7mo)
18	40	F	GNB	1	no		alive (7mo)
19	396	F	GNB Relapse		no		alive (2mo)
20	109	F	GN		no		alive(16mo)
21	56	F	GN		no		alive (7mo)
22	183	M	GN		no		alive (6mo)
23	34	M	GN		yes	no	alive (3mo)

[a]NB, neuroblastoma; GNB, ganglioneuroblastoma; GN, ganglioneuroma.

as in bone marrow (BM) stem cells and cytotoxic T-lymphocytes. P-gp is an ATP-dependent transport protein that extrudes a variety of chemotherapeutic drugs from the cell. It thus interferes with their intracellular accumulation and therefore diminishes their cytotoxicity (22). Vinca alkaloids, epipodophyllotoxins and anthracyclines, which are used in the treatment of NB, are substrates of P-gp action.

The elucidation of the clinical significance of P-gp expression in several human malignancies remains an important research goal as it might provide a target mechanism to overcome resistance by using specific agents that inhibit P-gp function (19). However, the extent to which P-gp expression plays a role in MDR in NB remains controversial.

Until now, the evaluation of P-gp expression in neuroblastic tumors has been based on different techniques, such as immunohistochemistry or immunocytochemistry, Northern blot, slot-blot, in situ hybridization, and reverse-transcriptase polymerase chain reaction (RT-PCR), resulting in conflicting data. Flow cytometry, extensively used to determine P-gp in hematological malignancies (26), offers the opportunity to identify tumor cells by using multiple color immunofluorescence. Moreover, functional flow cytometric testing provides the advantage of directly evaluating the transport capacity of P-gp and the inhibiting ability of reversal agents.

In the present study, we have determined the clinical value of both P-gp expression and function in 23 consecutive patients suffering from a neuroblastic tumor. Therefore, triple-color flow cytometry using CD45 and the monoclonal antibody NB84 that was raised to neuroblasts (36), and a functional test have been applied to dissociated neuroblastic tumors (22 samples) as well as to metastatic NB cells in the BM (four samples). The flow cytometric immunological results were compared with an immunocytochemical detection of P-gp in 16 patients and in six human NB cell lines.

MATERIALS AND METHODS

Cell Lines

Six human NB cell lines were studied: SK-N-SH (6), GI-ME-N (17), GI-LI-N (=IMR32) (37), NGP (8), TR14 (13), and UHG-PN (40). Cells were harvested by trypsinization. In the flow cytometric experiments, dead cells were excluded with propidium iodide staining.

Patient Samples

Twenty-three patients, including 22 children (mean age: 44 months; range: 0–183 months) and one adult, were studied. Clinical data of the patients are summarized in Table 1. Staging of the NB was done according to the International Neuroblastoma Staging System (INSS) (7).

In 21 patients (Table 2), single cell suspensions were prepared from 22 fresh tumor samples by collagenase disaggregation (1000 U/ml for 1 h at 37°C). Subsequently, cells were washed, resuspended and cell clumps were removed by filtering through a 70-μm nylon membrane. Five of these 22 samples were obtained after multi-chemotherapy including P-gp substrates.

In four children with NB stage 4, BM was analyzed at the onset (patient 1, 2, and 5) or after induction chemotherapy (patient 3). Mononuclear cells were isolated from the BM using Ficoll-Hypaque density gradient centrifugation.

Serial samples were available for two patients. From patient 8, a specimen of the primary tumor and one of the

Table 2
*Results of Flow Cytometric (FCM) Immunologic and Functional Assays, and of the Immunocytochemical (APAAP) Detection of P-gp**

Patient	Sample	FCM immunologic		FCM functional	APAAP	
		4E3 (MFI)	MRK16 (MFI)	(RR)	4E3	MRK16
1	BM	4.19	ND[c]	25.0	+	+
2	BM	0.80	1.09	0.83	−	−
3	BM[a]	0.98	0.94	NE[d]	ND	ND
	Tumor[a]	3.12	2.47	NE	ND	ND
4	Tumor	2.64	2.75	14.1	−	−
5	BM	1.16	1.32	0.71	NE	NE
	Tumor	1.33	0.97	NE	−	−
6	Tumor[a]	1.40	1.31	NE	ND	ND
7	Tumor	1.50	2.61	1.24	−	−
8	Tumor	2.84	2.82	2.46	−	−
	Tumor[a,b]	1.94	2.56	NE	−	−
9	Tumor	4.49	3.78	1.35	−	−
10	Tumor	2.59	2.65	2.75	+	+
11	Tumor	2.36	2.03	1.81	+	+
12	Tumor	7.32	9.08	5.05	ND	ND
13	Tumor	9.23	20.7	3.34	+	+
14	Tumor	2.27	2.79	NE	−	−
15	Tumor	1.32	1.22	1.10	−	−
16	Tumor[a,b]	0.88	0.73	NE	−	−
17	Tumor	23.6	23.1	1.76	−	−
18	Tumor	15.1	16.9	2.34	ND	ND
19	Tumor	18.2	12.4	1.26	+	+
20	Tumor	1.22	1.46	NE	ND	ND
21	Tumor	10.0	26.6	1.33	ND	ND
22	Tumor	3.34	4.97	1.10	ND	ND
23	Tumor[a]	6.98	5.47	0.91	+	+

*ND, not done; NE, not evaluable; MFI, mean fluorescence index; RR, Rho 123 retention ratio; +, positive; −, negative.
[a]Tested after chemotherapy.
[b]Relapse tumor.

relapse tumor after chemotherapy was analyzed. From patient 3, cells from a BM sample taken after one course of chemotherapy (including vincristine, cyclophosphamide, cisplatin, and etoposide) containing still 5% neuroblasts was used, as well as the patient's primary tumor which was removed after six courses of chemotherapy.

Flow Cytometry

Fluorescence was analyzed on a FACSort flow cytometer (Becton Dickinson, Erembodegem, Belgium) calibrated with CaliBRITE beads. The FACSort flow cytometer is equipped with an argon ion laser emitting a 488-nm beam at 15 mW as excitation source and 530/30, 585/42 nm band-pass filters, and a 650 long-pass filter. At least 10,000 events are counted and analyzed with Cell Quest software (Becton Dickinson, Erembodegem, Belgium).

Flow Cytometric Analysis of the NB84 Epitope

Both unfixed and fixed (2% formalin in acetone (FAC) for 10 s at room temperature) samples of NB cell lines and patients were evaluated.

Ten microliters of the monoclonal antibody NB84 (NCL-NB84-FITC, Novocastra Laboratories, Newcastle, UK), as delivered by the manufacturer, was added to 90 μl cell suspension (5×10^6 cells/ml) and incubated for 30 min at 4°C. Cells were washed twice and resuspended for analysis.

A mean fluorescence index (MFI) was determined by dividing the mean fluorescence of the cells stained with NB84 by that of cells stained with the isotype control antibody IgG_1 (Dako Corporation, Glostrup, Denmark).

Determination of P-gp Expression by Flow Cytometry

Two monoclonal antibodies which recognize different extracellular epitopes of P-gp, 4E3 and MRK16 (1,21), were used, as advised by the consensus recommendations on P-gp detection (4).

Cells (5×10^5) were incubated for 30 min at room temperature with a matched-isotype control antibody (IgG_{2a}, Dako Corporation, Glostrup, Denmark) and with either the monoclonal antibody 4E3 (7.5 μg/ml, Dako Corporation, Carpinteria, CA) or MRK16 (5 μg/ml, Kamiya Biomedical Company, Thousand Oaks, CA). Cells were washed twice with PBS-BSA-NaN_3, and then incubated for 20 min with phycoerythrin (PE)-labeled goat $F(ab')_2$ anti-mouse IgG (Caltag Laboratories, Burlingame, CA). Subsequently, cells were stained with the fluorescein isothiocyanate (FITC)-conjugated neuroblastoma marker NB84 and the peridinin chlorophyll protein (Per-CP)-conjugated CD45 (Becton Dickinson, Erembodegem, Belgium) for 30 min at 4°C. Cells were washed twice with PBS-BSA-NaN_3 and resuspended for analysis. A forward scatter threshold was set to exclude small cell fragments. NB84 positive/CD45 negative cells ($NB84^+$ /$CD45^-$) were gated to include the cell population of interest. No arbitrary cutoff points were used for assessing positivity (4,23). P-gp expression was presented as a ratio of the mean fluorescence intensity of the monoclonal antibody / mean fluorescence intensity of the isotypic control antibody (MFI).

Flow Cytometric Functional Analysis of P-gp

Functional analysis was carried out by studying the accumulation and retention pattern of 200 ng/ml rhodamine-123 (Rho 123, Sigma, St. Louis, MO), which is a P-gp substrate (9), in the absence and in the presence of verapamil (Isoptine, Knoll, Belgium) as described by others (15,27,28). Briefly, 10^5 cells were incubated for 1 h at 37°C with 200 ng/ml Rho 123, and with or without 10 μM verapamil. The intracellular Rho 123 accumulation was measured. Cells were washed twice with cold (4°C) Hank's balanced salt solution (HBSS) without phenol red and resuspended. Verapamil was added and the median fluorescence of the retained Rho 123 was determined after an efflux time of 1 h. Cells were considered to be functionally active if they exhibited a Rho 123 efflux, and if this efflux was inhibited by the presence of verapamil. The retention ratio (RR) was calculated by dividing the median Rho 123 fluorescence in the presence of verapamil by the median Rho 123 fluorescence in the absence of verapamil.

Table 3
*NB84 Reactivity and P-gp Expression and Function in Six Human NB Cell Lines**

Cell line	NB84 (MFI) without FAC	NB84 (MFI) with FAC	APAAP 4E3	FCM immunologic 4E3 (MFI)	FCM immunologic MRK16 (MFI)	FCM functional (RR)
1. GI-LI-N	1.76	1.81	−	1.00	1.10	0.84
2. UHG-PN	2.07	1.78	−	3.51	3.34	3.11
3. GI-ME-N	1.02	2.18	−	0.94	0.98	0.51
4. TR14	0.96	1.81	+	9.99	14.68	11.97
5. SK-N-SH	0.69	2.87	+	9.60	10.96	10.81
6. NGP	1.01	2.84	−	1.04	1.09	0.90

*FAC, 2% formalin/aceton permeabilization; MFI, mean fluorescence index; RR, Rho 123 retention ratio; +, positive; −, negative.

Determination of P-gp Expression by Immunocytochemistry

BM smears and tumor imprints were air-dried and kept frozen at −20°C until analysis. P-gp expression on neuroblastic cells was determined using an alkaline phosphatase-anti-alkaline phosphatase immunocytochemical assay (APAAP) as described previously (16). Both 4E3 and MRK16 monoclonal antibodies were used.

Control Experiments

Samples of the well-characterized human myeloid leukemia cell line K562 and its P-gp expressing resistant clone K562/VLB (38) were included in each flow cytometric and immunocytochemical experiment as negative and positive controls, respectively.

RESULTS

Evaluation of NB84 Reactivity in NB Cell Lines and Patient Samples

For the NB cell lines, two of six cell lines scored positive for NB84 without permeabilization (GI-LI-N and UHG-PN) whereas the others (SK-N-SH, GI-ME-N, TR14 and NGP) were negative (Table 3). Exposure to collagenase did not influence these findings (data not shown). After FAC permeabilization for 10 s at room temperature all cell lines scored positive. The MFI for NB84 reactivity in GI-LI-N and UHG-PN remained almost equal before and after permeabilization (Table 3). NB84 staining, compared to staining by the isotype matched control antibody IgG_1, before and after permeabilization in the cell lines GI-LI-N and NGP is illustrated in Figure 1.

The tumor cells in all but one patient samples scored clearly positive with NB84 without permeabilization, unlike the other nonmalignant cells that were negative. Bivariate plots of NB84 versus CD45 in a representative disaggregated tumor cell suspension and a BM sample are illustrated in Figure 2. In one patient with a histologically proven NB relapse (patient 8), NB84 reactivity remained negative after permeabilization. FAC permeabilization of the other patient samples enhanced the MFI, but always influenced unfavorably the scatter characteristics of the cells, as illustrated in Figure 3.

Flow Cytometric Determination of P-gp Status in K562 and K562/VLB Cell Lines

During analysis of all NB cell lines or patient samples, P-gp expression and function were determined in the P-gp

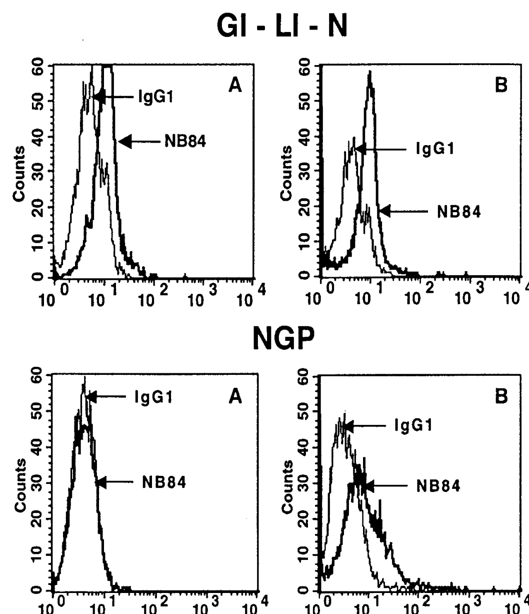

FIG. 1. The results of the staining of the neuroblastoma cell lines GI-LI-N and NGP with NB84 without permeabilization **(A)** and after permeabilization with 2% formalin/acetone (FAC) **(B)**. GI-LI-N shows a positive staining with NB84 in the unpermeabilized cells in contrast to NGP. Permeabilization does not influence the staining of GI-LI-N, whereas NGP cells become positive.

negative cell line K562, and in its sub-line, K562/VLB, which is selected from K562 by exposure to vinblastin (20 ng/ml) (38).

In K562 cells, the mean fluorescence ratio (± SD) for 4E3 and MRK16 compared to the isotypic control in 30 experiments was 1.01 ± 0.12 and 1.09 ± 0.15, respectively. In K562/VLB cells, the fluorescence intensity after staining with 4E3 or MRK16 was much brighter than the isotypic control antibody, resulting in mean (± SD) values of 85.6 ± 21.7 and 73.3 ± 25.2, respectively.

The Rho 123 RR, as determined by dividing the median Rho 123 values in the presence of verapamil by those in its absence, were 0.73 ± 0.08 and 4.00 ± 1.71 for K562 and K562/VLB, respectively. The Rho 123 RR, as determined by mean Rho 123 values, were 0.73 ± 0.08 and 5.03 ± 2.49 for K562 and K562/VLB, respectively. The Rho 123 accumulation and retention pattern in the absence or

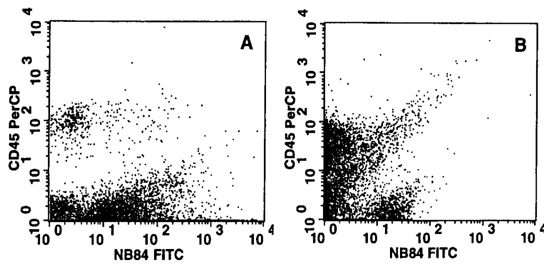

FIG. 2. Dot plots of CD 45 versus NB84 in a representative dissociated tumor sample (A) (patient 12) and in a BM sample (B) (patient 2).

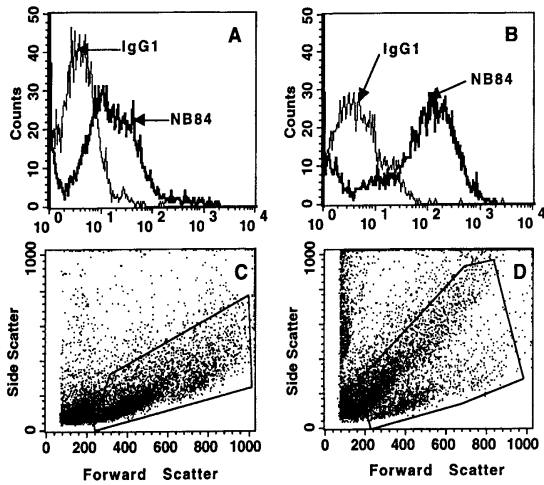

FIG. 3. NB84 fluorescence compared to the isotypic control IgG_1 in tumor cells of patient 13 before (A) and after permeabilization (B). The scattergram of this dissociated tumor sample (C) is influenced by the FAC permeabilization (D).

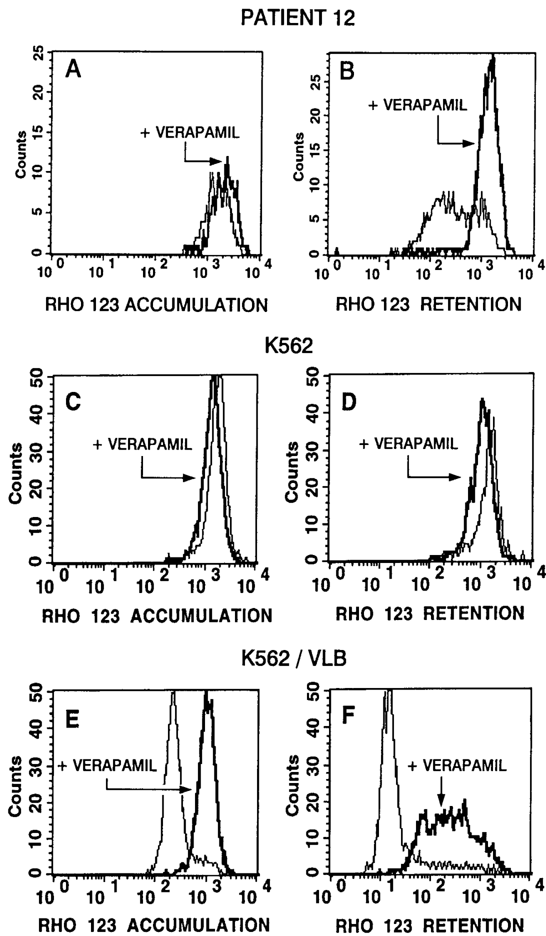

FIG. 4. The results of the flow cytometric functional assay in a representative tumor sample (patient 12) and in the control cell lines K562 and K562/VLB. Rho 123 accumulation (A, C, E) and retention (B, D, F) is shown in the absence (gray line) and presence (black line) of verapamil.

presence of verapamil for both control cell lines are illustrated in Figure 4C–F.

Flow Cytometric Determination of P-gp Status in NB Cell Lines

P-gp expression and function was determined in at least three different flow cytometric experiments. The cell lines SK-N-SH, TR14, and UHG-PN always had a positive P-gp expression with both 4E3 and MRK16. They exhibited Rho 123 efflux that was significantly inhibited by verapamil (Table 3). The three other cell lines (GI-LI-N, GI-ME-N, and NGP) always scored negative for both the immunologic and the functional test. The correlation coefficients between both monoclonal antibodies and between 4E3 or MRK16 and the functional test result were 0.98, 0.99, and 0.99, respectively.

Neither recognition of the P-gp epitopes by 4E3 and MRK16, nor P-gp function was impaired in the NB cell lines and in the control cell lines K562 and K562/VLB by exposure to collagenase (data not shown).

Flow Cytometric Determination of P-gp Status in Neuroblastic Patients

P-gp expression. P-gp expression was determined in $NB84^+/CD45^-$ cells of 23 patients (22 dissociated tumor samples and 4 BM samples containing NB cells). Triple-color flow cytometry yielded divergent MFI values, ranging from 0.80 to 23.6 and from 0.73 to 26.6, for 4E3 and MRK16, respectively (Table 2). The correlation coefficient between both monoclonal antibodies was 0.91. Each test result was compared with the positive and negative control cell line K562 and K562/VLB. In eight samples [patient 2, 3 (BM), 5 (BM and tumor), 6, 15, 16, 20], fluorescence intensities resemble the negative cell line K562 or are slightly higher. In the other samples, the fluorescence of the cells stained with 4E3 or MRK16 was remarkably higher compared to the ones stained with the isotype control antibody (mean fluorescence indices con-

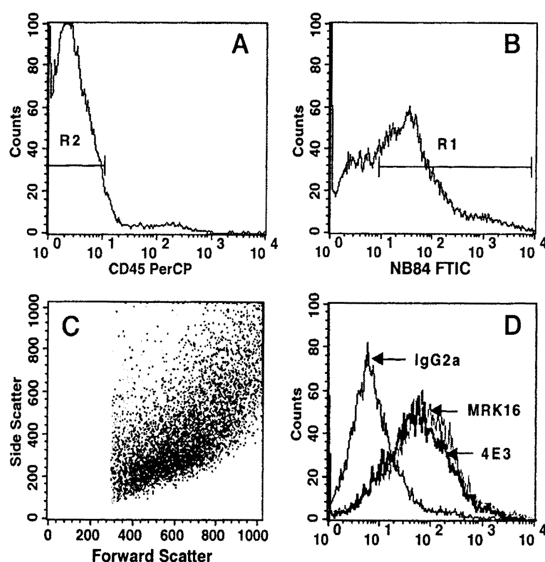

FIG. 5. Triple-color flow cytometric analysis of a single-cell suspension obtained from the NB of patient 12. The expression of CD45 and NB84 are shown in histogram **(A)** and **(B)**, respectively. Histogram **(D)** shows the positive staining with 4E3 and MRK16 compared to IgG_{2_a} of the (R1 and R2)-gated cells **(C)**.

sistently above 1.50). None of these samples had a fluorescence intensity as bright as the positive cell line K562/VLB.

There was no significant difference in P-gp expression between samples obtained prior or after exposure to chemotherapy (Table 2). We also found almost similar results for both 4E3 and MRK16 in the sequential samples of patient 8 (tumor at initial diagnosis and at relapse after chemotherapy).

Patient 3 was not tested prior to chemotherapy. A BM sample of this infant was analyzed after 1 month of polychemotherapy and contained only 5% tumor cells, as detected by the triple-parameter assay. This percentage residual neuroblasts was in agreement with the light microscopic examination of BM smears.

In these BM neuroblasts, P-gp was not detected. Tumor material after six courses of chemotherapy was evaluated with the triple-color assay and the MFI of 4E3 and MRK16 was 3.12 and 2.47, respectively.

Figure 5 shows the test result of the immunologic assay in a single-cell suspension obtained from the tumor of patient 12 (scattergram in Fig. 5C). The expression of CD45 and NB84 are shown in Figure 5A and B, respectively. Figure 5D shows the positive staining with 4E3 and MRK16 compared to IgG_{2_a} of the electronically gated ($CD45^-/NB84^+$) cells (Fig. 5C). The bivariate plot of CD45 versus NB84 of this sample is shown in Figure 2A.

P-gp function. Characteristics of positive cells are: an enhancement of the Rho 123 accumulation in the presence of verapamil; a Rho 123 retention that is consistently lower than the Rho 123 accumulation value; and an enhancement of the Rho 123 retention when verapamil is added to the sample. An example of a positive functional test result in a patient sample (patient 12) is shown in Figure 4A and B.

In eight samples, the functional assay could not be evaluated because the number of tumor cells was too low or because the tumor cells could not be identified on scatter characteristics (Table 2, NE = not evaluable). The Rho 123 retention ratios of the other 18 samples varied from 0.71 to 25.0. In 13 of these 18 samples (patient 1,4,7,8,9-13,17-19,21), enhancement of Rho 123 accumulation was consistently seen when verapamil was added and a significant Rho 123 efflux was observed that was inhibited by the presence of verapamil. In the other five samples, the Rho 123 RR ranged from 0.71 to 1.10. There was a lack of correlation between the MFI for 4E3 or MRK16 and the Rho 123 retention ratios (correlation coefficient R = −0.12 and −0.07, respectively). The most discrepant test results were found in stage 4 NB with the highest RR, and in the well-differentiated tumors with high MFI values and concomitant low RR.

Clinical Value of P-gp Expression and Function

Clinical information of the 23 patients is presented in Table 1. Prognostic unfavorable features, such as older age and advanced stage, were not associated with high 4E3 or MRK16 fluorescence. The highest P-gp expression levels (with MFI values above 9) were found in six patients (patient 12, 13, 17, 18, 19, 21), which suffer from NB stage 1, GNB or GN. In the group of NB stage 4 patients, four of six patients had expression levels of at most 1.40. Patient 16, with a localized NB relapse, also had low MFI values (0.88 and 0.73). Our data indicate a discordance between expression level and activity: the highest Rho retention ratios were observed in patient 1 and 4, which suffer from NB stage 4; the GNB patients, on the other hand, with high expression levels, had a RR between 1.26 and 2.34.

During our study period, patient 1 experienced a bad outcome. This child, with a 4E3 fluorescence index of 4.19 and a Rho 123 RR of 25.03, was refractory to chemotherapy and died. In the other patients, no apparent association was seen between P-gp expression or function and the eventual clinical outcome.

Consecutive samples were obtained in two patients. Patient 3 was analyzed at the beginning and the end of chemotherapy. The P-gp expression level of his neuroblasts raised from 0.98 and 0.94 to 3.12 and 2.47, respectively, which might point to the induction of P-gp expression by prolonged exposure to cytostatic agents. This was, however, not the case for patient 8, who was tested at initial diagnosis and at relapse after chemotherapy.

Correlation of Flow Cytometric Immunological Test and Immunocytochemical APAAP Technique

Cytospin samples of 6 human NB cell lines, 15 tumor imprints of 14 different NB patients, and 2 BM smears, metastatically invaded by NB cells, were analyzed using 4E3 and MRK16 in an immunocytochemical APAAP technique (Tables 2 and 3).

Two of the three flow cytometric positive cell lines (SK-N-SH and TR14) had a positive immunocytochemical staining; all other cell lines scored negative.

In 10 of 17 patients, the results were in concordance with the immunological flow cytometric assay. In seven samples, there was no reactivity seen in the APAAP assay, while flow cytometry yielded a positive result. However, no statistically significant correlation between these test results in our patient group could be found ($\chi^2 = 1.19$, $P = 0.28$).

DISCUSSION

Patients suffering from neuroblastic tumors are currently being classified into prognostic subsets based on different clinical and biologic features. In this study, a triple-color and a functional flow cytometric assay were applied to NB cell lines and patients with a neural crest tumor.

So far, only expression rates of P-gp in NB specimens are reported in literature, and they vary from study to study. This is due in part to the use of nonstandardized techniques and study protocol designs that cannot be compared. Chan (11) used an immunocytochemical assay with enhanced sensitivity and reported a significant relationship between P-gp expression at diagnosis and prediction of therapeutic success or failure. Bénard (5), investigating MDR1 overexpression by Northern blot hybridization, found by multivariate analysis a significant association with increased mortality risk. Others (3,30) failed to reproduce these results and found a marked increase in MDR1 expression following chemotherapy, which also induced differentiation of the tumor cells. Favrot (18) and O'Meara (32) were unable to detect P-gp in NB cells, as it was seen in non-neoplastic stromal cells only. The results of a recent study by Haber et al. (20) suggest a clinical role for the *MDR1* gene in specific subgroups of primary NB, as identified by absence of *MYCN* gene amplification and expression of the multidrug resistance-associated protein (*MRP*) gene. An immunocytochemical study, performed earlier in our hospital (16) showed a correlation of P-gp expression with nonlocalized disease and with poor differentiation, but failed to indicate any prognostic significance as a predictor of recurrence or progressive disease.

Disadvantages of these assays include application of the blotting and PCR techniques to bulk tumor homogenates; failure to differentiate tumor from normal cells possibly expressing P-gp; inability to detect small subpopulations below the threshold of recognition yet contributing to clinical drug resistance; and lack of sensitivity and objective interpretation of microscopic techniques, irrespective of their strength in differentiating various cell types (9,12).

Flow cytometry can rapidly identify heterogeneous tumor populations based on fluorescent drug content or marker expression. Large numbers of cells can be analyzed and, using a panel of antibodies, multiparameter investigations are possible. Flow cytometric functional assays offer relevant information on P-gp mediated MDR since the effect of P-gp inhibitors can be checked. Recently, we have applied the flow cytometric functional test to check on the imaging results obtained with ^{99m}Tc-Sestamibi, a tracer which is recommended as an in vivo examination of the P-gp status of tumors (14).

Discerning neoplastic from normal cells in a neuroblastic cell suspension is a foremost prerequisite in order to obtain a specific identification of multidrug resistant, P-gp expressing NB cells. Therefore, we have used the light-scattering characteristics and CD45- and NB84-reactivity to identify neuroblasts in BM and enzymatically dissociated tumor specimens. NB cells consistently lack CD45 expression (25). The murine monoclonal antibody NB84, now commercially available for flow cytometric investigation as an FITC-conjugated marker, recognizes an unknown antigen of 57 kDa. It reacts immunohistochemically with NB, but rarely also with other small round cell tumors (e.g., Ewing sarcoma and medulloblastoma) and/or some epithelial cells. Normal lymphoid tissues are negative (29,36).

In our experimental setting, two of six nonpermeabilized NB cell lines, and all but one NB patient samples stained positive with NB84. Prior subjection to the FAC permeabilization resulted in NB84-reactivity in all NB cell lines. The patient sample remained negative. The fluorescence intensity of NB84 in the other patient samples was enhanced by permeabilization, which increased the discrimination of tumor cells, but deteriorated their scatter characteristics.

Our data, which demonstrate very low MFI values in 4 of 6 stage 4 NB patients and the highest P-gp expression levels in stage 1 NB, GNB and GN, support the findings of several authors (3,24,30,31,33), who reported on an increased P-gp expression in localized and more differentiated tumors. Since P-gp is consistently expressed in adrenal glands of adults, whereas it is absent in the same organ of fetuses and newborns (35), Obana et al. (31) hypothesized that P-gp expression in sympathetic ganglia cells reflects their normal maturation.

We found a good correlation between immunologic and functional tests in six NB cell lines. In our patient population, however, we observed an important dissociation between the flow cytometric P-gp expression level and activity value. This is in accordance with Bates et al. (2), who described an increased P-gp expression without a concomitant decrease in drug accumulation in NB cell lines that were previously treated with differentiating agents.

CONCLUSIONS

In the present study, we have demonstrated that multiparameter flow cytometry offers an easy, specific, and sensitive method, recognizing neuroblasts and allowing the evaluation of P-gp expression. It identifies target cells that can be tested functionally. Proof has been provided that the method of investigation is applicable to cultured NB cell lines as well as to neuroblasts obtained from clinical specimens, such as BM and disaggregated tumor cells. Low P-gp activities were found in differentiated tumors, whereas a high P-gp activity level was seen in some clinically important stage 4 patients. Our results suggest that these flow cytometric assays are useful in

selecting those patients in whom it may be beneficial to treat with P-gp reverting agents.

ACKNOWLEDGMENTS

We thank Dr. Frank Speleman (Department of Medical Genetics, University Hospital Gent) for technical assistance (preparation of single cell suspensions) and for the provision of the NB cell lines SK-N-SH, NGP, UHG-PN, GI-LI-N, GI-ME-N and TR14. We are also grateful to Jeanine Geerinck and Brendy Van Butsel for skillful assistance in the immunocytochemical assays.

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TC-99M SESTAMIBI IMAGING IN PEDIATRIC NEUROBLASTOMA AND GANGLIONEUROMA AND ITS RELATION TO P-GLYCOPROTEIN

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European Journal of Nuclear Medicine 1999; 26: 396-403

Original article

Technetium-99m sestamibi imaging in paediatric neuroblastoma and ganglioneuroma and its relation to P-glycoprotein

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Received 17 October and in revised form 13 December 1998

Abstract. Imaging with technetium-99m sestamibi offers a non-invasive approach to detect the presence of functional P-glycoprotein (Pgp), one of the major causes of multidrug resistance, in human malignancies. A clinical role for Pgp has been suggested in the subpopulation of primary neuroblastoma without amplification of the proto-oncogene MYCN. We wanted to evaluate the usefulness of ^{99m}Tc-sestamibi scintigraphy in the screening of neural crest tumours for the presence of Pgp. In ten children suffering from MYCN-negative neuroblastoma, ganglioneuroblastoma or ganglioneuroma, ^{99m}Tc-sestamibi imaging was performed at initial diagnosis. All patients underwent planar imaging 20–30 min and 3.5–4 h after intravenous injection of 740 MBq/1.73 m^2 ^{99m}Tc-sestamibi. Tumour to normal tissue ratios, as well as washout rates, were determined and compared with in vitro flow cytometric analysis of Pgp expression and function. Pgp expression was analysed flow cytometrically with the monoclonal antibodies 4E3 and MRK16, and Pgp function was evaluated by means of rhodamine 123 uptake and efflux either in the absence or in the presence of the Pgp inhibitor verapamil. In nine of ten patients, we found that the intratumoral ^{99m}Tc-sestamibi activity was comparable to the background activity, which might be suggestive of Pgp presence. This was confirmed flow cytometrically in all but one patient. ^{99m}Tc-sestamibi enhancement was seen in the primary tumour and the bone marrow metastases of one of the ten patients, and this result was concordant with a negative Pgp status. The findings presented suggest that ^{99m}Tc-sestamibi imaging results might correlate with the presence of functional Pgp in neural crest tumours without MYCN amplification.

Key words: Technetium-99m sestamibi – P-glycoprotein – Neuroblastoma – Ganglioneuroma – Flow cytometry

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Eur J Nucl Med (1999) 26:396–403

Introduction

Neuroblastoma (NB), a childhood tumour originating in neural crest cells, has a variable clinical behaviour. While maturation into benign ganglioneuroma (GN) and even spontaneous regression have been observed, the long-term survival of children with extensive disease [International Neuroblastoma Staging System (INSS) stages 3 and 4] is poor and has not improved significantly during the last 10 years [1]. The major cause of treatment failure in NB is the occurrence of resistance to multiple cytotoxic drugs, also called multidrug resistance or MDR [2]. MDR is frequently associated with amplification or increased expression of the MDR1 gene and overproduction of its product, P-glycoprotein (Pgp) [3]. More recently, the multidrug resistance-associated protein (MRP) has been identified as an alternative cause of MDR [4]. These energy-dependent transmembranous pumps extrude a variety of chemotherapeutic drugs out of the cell, thus interfering with their intracellular accumulation and hence diminishing their toxicity. Cytotoxic agents commonly used in the treatment of advanced NB, such as epipodophyllotoxins, vinca alkaloids and anthracyclines, are substrates of Pgp and MRP action [3, 5]. Both Pgp and MRP can be used as target mechanisms in overcoming drug resistance by using agents that inhibit Pgp function [6] or by the application of cytotoxic agents without affinity for MRP [7].

NB is a heterogeneous disease. Prognostic subgroups can be defined on the basis of various clinical and biological features, which subsequently allows the development of risk-related therapy [8]. Amplification of the MYCN proto-oncogene, which occurs in 30%–40% of patients with advanced disease and in 5%–10% of children with low stages of the disease, is one of the most powerful indicators of both response to chemotherapy

European Journal of Nuclear Medicine
Vol. 26, No. 4, April 1999 – © Springer-Verlag 1999

and poor outcome in NB [1]. Recently, evidence has been provided that MYCN regulates the expression of the MRP gene in NB [9]. Consequently, MRP expression is a strong parameter of adverse outcome in NB [10].

The extent to which Pgp expression plays a role in MDR in NB remains controversial. Chan et al. [11] reported a significant relationship between Pgp expression at diagnosis and prediction of success or failure of therapy, and in multivariate analysis Bénard et al. [12] found a significant association with increased mortality risk. Others, however, have failed to reproduce these results [13–15], and some even were unable to detect Pgp in NB cells [16, 17]. These discrepancies are probably the result of non-standardised detection methods, the application of incomparable study design protocols and different sample selection criteria (before or after exposure to chemotherapeutic agents) [18]. Haber et al. [19] reported a significant association between high MDR1 expression in NB patients without amplification of the MYCN gene and poor outcome. These findings suggest prognostic significance for Pgp in MYCN-negative NB subpopulations.

In 1993, Piwnica-Worms et al. [20] reported that ^{99m}Tc-sestamibi is a substrate for Pgp. The correlation of ^{99m}Tc-sestamibi uptake and efflux characteristics with Pgp presence has been observed in vitro [20–22], as well as in vivo in nude mice with solid tumour xenografts [20] and in several human adult tumour types such as breast and lung cancer [23–26].

The aim of this study was to evaluate the suitability of ^{99m}Tc-sestamibi scintigraphy for in vivo evaluation of the presence of functional Pgp in neural crest tumours. Therefore, ^{99m}Tc-sestamibi imaging was performed in ten patients [seven with NB, two with GN and one with a ganglioneuroblastoma (GNB)] at initial presentation. The scintigraphic results were compared with the in vitro flow cytometric detection of both Pgp expression and function.

Materials and methods

Patient and tumour characteristics

Ten children (mean age: 30.4 months; range: 0–109 months) with the diagnosis of neural crest tumour (NB, GN, GNB), were studied. Informed consent was obtained from all patients and their parents. Patient characteristics are listed in Table 1. All patients underwent computed tomography (CT)/magnetic resonance imaging (MRI) scanning of the primary tumour sites as well as meta-iodobenzylguanidine (MIBG) and ^{99m}Tc-sestamibi imaging at initial presentation. The size of the primary tumour (Table 1) was assessed on CT, employing the largest diameter in centimetres with verification by surgery reports and histological data when available. Areas of necrosis were not observed on imaging studies in any of the tumours. Contrast enhancement was seen in all tumours on CT and MRI scans. Staging of the NB was done according to the INSS [27]. On bone marrow examination, 42%, 44% and 2% neuroblasts were found in patients 1, 2 and 3, respectively. Within 2 weeks after admission to the hospital, biopsy (patients 3–5) or resection (patients 6–10) of the primary tumour was performed to confirm the diagnosis histologically and to obtain samples for determination of biological parameters (MYCN amplification, 1p deletion, DNA-ploidy). In all samples, viable tumour tissue was seen.

NB was defined histologically as a tumour composed predominantly of neuroblasts, sometimes displaying a variable degree of ganglionic differentiation. GNB was defined as a tumour with the appearance of an NB as described above but which in addition contained a predominant ganglioneuromatous stroma with Schwann cells. GN was defined as a fully differentiated tumour that contained no immature elements [28].

For the determination of MYCN amplification (number of copies >6), fluorescence in situ hybridisation (FISH) of nuclei from tumour cells was performed with the MYCN probe (LSI N-myc cat.no 32-190000-100, Vysis). FISH was performed as described previously [29]. In none of the patient samples was MYCN amplification found.

Expression of MRP was determined on stored bone marrow smears or tumour imprints, using the monoclonal antibody MRPm6 and an immunocytochemical APAAP technique, as described previously [13,30] (Table 1).

Imaging

^{99m}Tc-sestamibi imaging. Guided spot images and whole-body images (scanning speed 13 cm/min), were obtained 20–30 min (equilibrium phase, as described previously [24, 26]) and 3.5–4 h following the injection of 740 MBq/1.73 m^2 ^{99m}Tc-sestamibi using a single-headed Toshiba gamma camera (GCA 901 A) equipped with a low-energy high-resolution collimator. For image processing, data were transferred to a Hermes processing system. The parameters investigated on the ^{99m}Tc-sestamibi images were tumour-to-background ratios (TM/BKG) and tumour and normal

Table 1. Patient and tumour characteristics

Patient[a]	Diagnosis[b]	Site[c]	Stage[d]	MIBG[e]	Size[f]	MRP[g]
1/B/35	NB	Adr	4	+	1.8/1.8/1.6	–
2/B/11	NB	Adr	4	+	5.0/3.0/6.4	ND
3/G/15	NB	Abd	4	+	6.5/6.9/8.0	+
4/G/0	NB	Cerv	3	–	5.0/3.0/3.5	–
5/B/17	NB	Abd	3	+	13.0/13.0/11.0	–
6/B/3	NB	Adr	1	+	3.3/2.1/3.0	–
7/G/16	NB	Abd	1	+	8.0/6.0/7.0	–
8/G/42	GNB	Abd	1	–	7.2/6.7/6.0	–
9/G/56	GN	Abd		+	2.0/4.4/7.0	NE
10/G/109	GN	Abd		–	10.4/8.0/7.0	–

[a] Patient number/B, boy; G, girl/age in months at diagnosis
[b] Diagnosis: NB = neuroblastoma; GNB = ganglioneuroblastoma; GN = ganglioneuroma
[c] Primary tumour localisation (Adr, adrenal; Abd, abdominal; Cerv, cervical)
[d] INSS tumour stage [27]
[e] MIBG uptake in the primary tumour (and BM metastases in patients 1–3)
[f] Tumour size in centimetres
[g] MRP expression: –, negative; +, positive; ND, not done; NE, not evaluable

tissue percentage washout rate per hour (%W-O/h). Tumour-to-background ratios were calculated by drawing a region of interest (ROI) over the primary tumour and one over the contralateral side, excluding normal physiological excretory activity. Additionally, an ROI was drawn over each bone marrow metastasis in patients 1–3, and an average value was determined. In the absence of visually assessable ^{99m}Tc-sestamibi uptake, either aligned MIBG images or CT data were used to determine the tumour location. Tumour and normal tissue %W-O/h were calculated on aligned (Hermes software, Nuclear Diagnostics, Sweden) early and late spot images, allowing the number of pixels in the ROI to remain unchanged for calculating the %W-O/h. After decay correction of the mean counts in the ROIs drawn on delayed images, the %W-O/h was calculated for primary tumours, bone marrow metastases and normal tissues using the formula:

$$\%\mathrm{W-O/h} = \frac{N(\mathrm{early}) - DN(\mathrm{delayed})}{N(\mathrm{early}) \times T} \times 100,$$

where N (early) represents the mean counts in the early image, DN (delayed) represents the decay-corrected mean counts in the delayed images and T represents the time interval (in hours) between early and delayed images. ^{99m}Tc-sestamibi scans were interpreted by two nuclear medicine physicians, unaware of the patients' clinical histories and flow cytometric findings.

^{123}I-MIBG scintigraphy. In all patients, MIBG images were obtained within 1 week of the ^{99m}Tc-sestamibi examination. The thyroid gland was blocked with potassium iodide. Twenty-four hours after the intravenous administration of 185 MBq/1.73 m^2, patients were scanned using a dual-headed gamma camera (Helix, Elscint, Israel) equipped with medium-energy all-purpose collimators. When judged necessary by a nuclear medicine physician, 48-h images were also obtained. Either whole-body images (anterior and posterior scanning speed 10 cm/min) or composite whole-body scans (±500 000 counts per image) were acquired, depending on the age of the child.

Determination of Pgp expression and function by flow cytometry

Flow cytometric experiments require sampling of tumour material and preparation of single cell suspensions. In two children with NB stage 4 (patients 1 and 2), mononuclear cells were isolated from the invaded bone marrow using Ficoll-Hypaque density gradient centrifugation. Patient 1 had 42% of neuroblasts in the bone marrow at initial presentation. From patient 2, no cells were available prior to chemotherapy; cells from a bone marrow sample taken after one course induction chemotherapy (including vincristin, cyclophosphamide, cisplatin and etoposide) were used. The latter sample still contained 5% neuroblasts (Table 2).

Within 2 weeks after admission, fresh specimens of the primary tumours of patients 3–10 were disaggregated by collagenase (1000 U/ml for 1 h at 37°C) to obtain single tumour cell suspensions. Subsequently, cells were washed and resuspended, and cell clumps were removed by filtering through a 70-μm nylon membrane. The primary tumour of patient 2 was removed after six courses of chemotherapy and disaggregated by collagenase.

For the flow cytometric determination of Pgp expression, a triple-colour assay was applied. Cells (5×10^5) were incubated for 30 min at room temperature with a matched-isotype control antibody (IgG2a, Dako Corporation, Glostrup, Denmark) or with either the monoclonal antibody 4E3 (7.5 μg/ml, Dako Corporation, Carpinteria, USA) or MRK16 (5 μg/ml, Kamiya Biomedical Company, Thousand Oaks, USA), which recognise different external epitopes of Pgp. Cells were washed, incubated for 20 min with phycoerythrin (PE)-labeled goat F(ab')$_2$ anti-mouse IgG (Caltag Laboratories, Burlingame, UK), and subsequently stained for 30 min at 4°C with the fluorescein isothiocyanate (FITC)-conjugated neuroblastoma marker NB84 [31, 32] and the peridinin chlorophyll protein (Per-CP)-conjugated CD45 (Becton Dickinson, Erembodegem, Belgium), a pan-leucocyte marker which is absent on NB cells [33]. Cells were washed twice and resuspended for analysis. NB cells were identified reliably after electronic gating to include NB84-positive and CD45-negative cells. Fluorescence was analysed on a FACSort flow cytometer (Becton Dickinson, Erembodegem, Belgium) with an argon ion laser emitting a 488 nm beam at 15 mW as excitation source, 530/30 and 585/42 nm band-pass filters, and a 650 long-pass filter. At least 10 000 events were counted. Cells were considered to be positive if their MFI (mean fluorescence intensity after staining with 4E3 or MRK16 divided by mean fluorescence intensity after staining with IgG2a) was at least 1.10 [34] (Table 2).

Functional flow cytometric analysis was carried out by studying the accumulation and retention pattern of 200 ng/ml rhodamine 123 (Rho 123, Sigma, St. Louis, USA), a fluorescent dye which is substrate of Pgp [35], either in the absence or in the presence of 10 μM verapamil (Isoptine, Knoll, Belgium), a well-known Pgp inhibitor, as described by others [36, 37]. Cells were considered to be functionally active if they exhibited a Rho 123 efflux and if this efflux was inhibited by the presence of verapamil, which is reflected in a Rho 123 retention ratio (median Rho 123 fluorescence in the presence of verapamil/median Rho 123 fluorescence in the absence of verapamil) greater than 1.10 [34].

Samples of the human myeloid leukaemia cell line K562 and its Pgp-expressing resistant clone K562/VLB were included in each flow cytometric experiment as negative and positive controls, respectively.

Statistical analysis

The significance of correlations between numerical data was determined by means of the Spearman rank correlation test.

Results

Flow cytometric results

Flow cytometric results are shown in Table 2. All disaggregated primary tumour samples (patients 2–10) showed increased Pgp expression as evidenced by their mean fluorescence intensity (MFI) after staining with 4E3 or MRK16 larger than 1.10. In the NB cells in the bone marrow of patients 1 and 2, MFI values for 4E3 and MRK16 staining inferior to 1.10 were found. The overall correlation between both monoclonal antibodies was very good ($r = 0.91$, $P = 0.004$).

Although the samples of patient 2 and patient 10 contained sufficient NB and GN cells, respectively, for immunological Pgp detection by the triple-colour assay, reliable results could not be obtained in the functional test (Table 2, NE=not evaluable). In eight patients (patients 1, 3–9) positive or negative Rho 123 retention ratios concurred with the respective positive or negative Pgp expression values. High Pgp expression levels were

Table 2. Correlations between tumour-to-background ratios, washout rates and flow cytometric Pgp expression and function

Patient No.	TM/BKG ratios[a]	Tumour %W-O/h[b]	Normal tissue %W-O/h[c]	Sample[d]	FCM funct[e]	FCM 4E3[f]	FCM MRK16[f]
1	1.0	4%	9%				
	1.2*	6%*		BM(42%)/I	−(0.83)	−(0.80)	−(1.09)
2	5.2	14%	10%				
	2.1*	0.3%*		BM(5%)/Ch	NE	−(0.98)	−(0.94)
				Tumour/Ch	NE	+(3.12)	+(2.47)
3	1.1	4%	10%	Tumour/I	+(14.07)	+(2.64)	+(2.75)
	1.2*	5%*					
4	1.0	2%	14%	Tumour/I	+(2.46)	+(2.84)	+(2.82)
5	1.0	5%	13%	Tumour/I	+(1.24)	+(1.50)	+(2.61)
6	0.9	3%	12%	Tumour/I	+(3.34)	+(9.23)	+(20.71)
7	0.7	2%	8%	Tumour/I	+(2.75)	+(2.59)	+(2.65)
8	1.2	6%	13%	Tumour/I	+(1.76)	+(23.55)	+(23.10)
9	0.9	4%	9%	Tumour/I	+(1.33)	+(10.04)	+(26.55)
10	0.9	6%	8%	Tumour/I	NE	+(1.22)	+(1.46)

[a] Tumour-to-background ratio of the primary tumour (BM metastases* in patients 1–3)
[b] Tumour % washout per hour of the primary tumour (BM metastases* in patients 1–3)
[c] Normal tissue % washout per hour
[d] Sample: BM, bone marrow (% NB cells); Tumour/I, at initial presentation; Tumour/Ch, after chemotherapy
[e] Result of flow cytometric functional test (Rho 123 retention ratio): –, negative; +, positive; NE, Not evaluable
[f] Result of flow cytometric immunological test (mean fluorescence index of 4E3 or MRK16): –, negative; +, positive

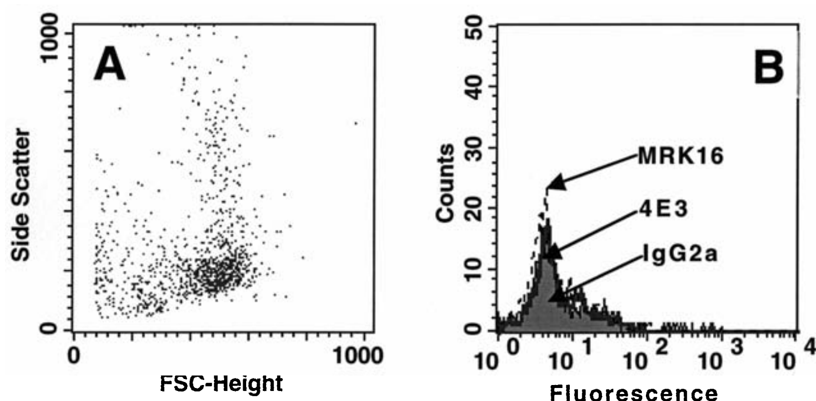

Fig. 1A, B. Result of the flow cytometric immunological assay in patient 2 (bone marrow sample containing 5% NB cells). **A** Scattergram of NB84-positive/CD45-negative cells. In the overlaid histogram (**B**), the horizontal axis represents the cellular fluorescence and on the vertical axis, numbers of cells are recorded. The mean fluorescence of the cells after staining with 4E3 (*black line*) and MRK16 (*dotted line*) equals the fluorescence of the cells stained with the isotypic control IgG2a (*grey fill-in*), which results in an MFI<1.10

found in patients 6, 8 and 9, who suffered from NB stage 1, GNB and GN, respectively, whereas a high Rho 123 retention ratio was observed in patient 3, who had an NB stage 4.

Figure 1 shows the results of the triple-colour flow cytometric assay in the bone marrow sample of patient 2, in whom no Pgp expression could be detected. Figure 2 shows the results of the triple-colour test and of the flow cytometric functional test in the tumour cell suspension of patient 7, and illustrates the presence of functionally active Pgp.

^{99m}Tc-sestamibi imaging results

All patient data are listed in Table 2. TM/BKG ratios, as determined on the early images, varied from 0.7 to 5.2 (mean 1.42, SD 1.19). In all but one primary tumours, intratumoral activity (ranging from 0.7 to 1.2) was comparable to the background activity, which might be suggestive for the presence of functional Pgp. By contrast, in the primary tumour and the occipital bone marrow metastasis of patient 2, TM/BKG ratios were 5.2 and 2.1, respectively, and ^{99m}Tc-sestamibi activity was visually discernible. ^{99m}Tc-sestamibi enhancement in this patient corresponded with flow cytometric absence of Pgp expression in NB cells from the invaded bone marrow.

Tumour %W-O/h varied from 0.3% to 14% (mean 4.7%, SD 3.3%), whereas normal tissue %W-O/h varied from 8% to 14% (mean 10.6%, SD 2.2%). No statistically significant correlations were found between the TM/BKG ratios and the tumour %W-O/h compared with the level of Pgp expression (4E3 and MRK16) or function as determined by flow cytometry.

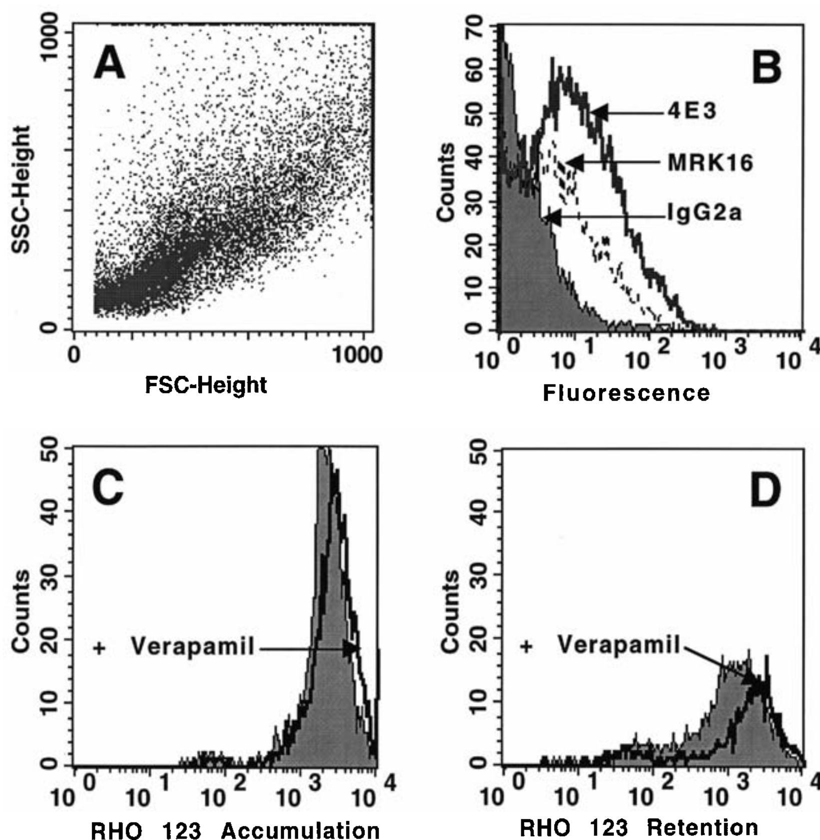

Fig. 2. Results of the flow cytometric immunological (**B**) and functional (**C**, **D**) assay in patient 7. **A** Scattergram of NB84-positive/CD45-negative cells. In the histograms (**B–D**), the horizontal axis represents the cellular fluorescence and on the vertical axis, numbers of cells are recorded. In the overlaid histogram (**B**), an enhancement of mean fluorescence after staining with 4E3 (*black line*) and MRK16 (*dotted line*) compared with the isotypic control IgG2a (*grey fill-in*) is observed, which results in an MFI>1.10. Rho 123 accumulation (**C**) and Rho 123 retention (**D**) are shown in the absence (*grey fill-in*) and presence (*black line*) of verapamil. Rho 123 accumulation enhances in the presence of verapamil (**C**). Rho 123 efflux is reversed by the presence of verapamil (**D**), resulting in a Rho 123 retention ratio >1.10

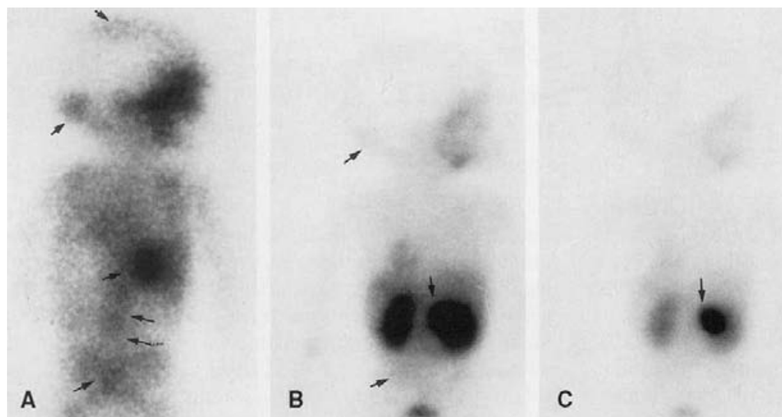

Fig. 3. MIBG scan (**A**), and early (**B**) and delayed (**C**) ^{99m}Tc-sestamibi scans of patient 2, an 11-month-old boy presenting with an NB stage 4. Both the primary MIBG-positive adrenal tumour and the bone marrow metastases (some of them indicated by *small arrows*) are ^{99m}Tc-sestamibi positive

Figure 3 shows the MIBG scan and the early and delayed ^{99m}Tc-sestamibi images of patient 2, a boy of 11 months old with an NB stage 4, at initial diagnosis. Both the primary adrenal tumour and the bone marrow metastases show ^{99m}Tc-sestamibi activity. Using the monoclonal antibodies 4E3 and MRK16, no Pgp was found in the bone marrow neuroblasts of this child after one course of chemotherapy (Fig. 1). After six courses of chemotherapy (no ^{99m}Tc-sestamibi scan available), the primary tumour was resected and a positive Pgp expression was found flow cytometrically.

Figure 4 illustrates the 4-h ^{99m}Tc-sestamibi findings in patient 7, a girl with an NB stage 1 located in the abdomen. The corresponding MIBG image, allowing as-

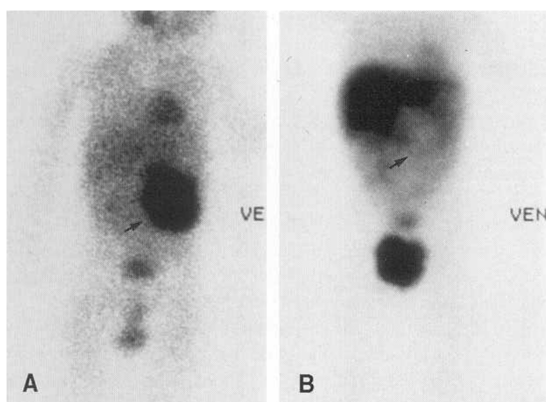

Fig. 4. A large abdominal MIBG-positive NB in patient 7, a girl with an NB stage 1 (**A**). On delayed ^{99m}Tc-sestamibi imaging (**B**) as well as on early scans (not shown), no ^{99m}Tc-sestamibi uptake was seen in the abdominal tumour

sessment of the tumoral burden, is also shown. Pgp was found in this patient using both flow cytometric immunological and functional assays (Fig. 2).

Discussion

Neuroblastic tumours are currently being classified into subsets with distinct biological characteristics and clinical behaviour. Identification of these subsets allows accurate prediction of outcome and may serve as a guide in the selection of appropriate treatment [8]. The contribution of Pgp to the multidrug-resistant phenotype in NB is unclear, but a clinical role for Pgp has been suggested in the MYCN-negative subgroup of primary NB [19]. Since Pgp inhibitors such as PSC 833 might be introduced in the chemotherapeutic regimen of NB patients, clinical studies revealing the exact significance of Pgp functionality in NB are warranted.

The in vitro assays which have been applied to characterise the Pgp status of tumours have some major drawbacks, limiting their clinical usefulness [18]. Blotting and PCR techniques are semi-quantitative and are applied to bulk tumour homogenates, which compromises the distinction of tumoral cells from normal stromal cells, possibly expressing Pgp. Immunological assays appear to have a low sensitivity when applied to cells expressing only low or moderate levels of Pgp. The interpretation of immunohistochemical results is labour-intensive and prone to subjective interpretation. Flow cytometric tests, although allowing accurate identification of NB cells and evaluation of both Pgp expression and function, require solid tumour disaggregation. Hence, the reliability of test results depends on the quality of the tumour specimen and on the amount of viable NB cells. Moreover, all in vitro assays are dependent on sampling of tumour material and hence may be influenced by chemotherapeutic treatment, which may cause necrosis or induce differentiation. The discrepancies between Pgp expression in the bone marrow sample of patient 2 and in the NB cells of his primary tumour might be explained by prolonged exposure to cytostatic agents which are able to induce Pgp expression.

^{99m}Tc-sestamibi scintigraphy is a non-invasive technique that can be performed at initial diagnosis, before the start of chemotherapy. Washout rates of the radiopharmaceutical product quantify the potential to transport the substrate out of the entire tumour burden and hence indicate the presence of a functional efflux pump [38]. In breast carcinoma and lung tumours, ^{99m}Tc-sestamibi scintigraphy has demonstrated its usefulness in establishing the in vivo Pgp status [23–26]. Moreover, it holds promise for the in vivo evaluation of the Pgp inhibitor PSC 833 in solid tumours [39, 40].

We prospectively studied the degree of ^{99m}Tc-sestamibi accumulation and its washout in neural crest tumours at initial presentation. We compared these imaging results with the flow cytometric level of Pgp expression and function. In the patient group presented, we found the highest tumour-to-background ratios in the primary tumour and the bone marrow metastases of patient 2, the only child in whom ^{99m}Tc-sestamibi activity was observed visually.

In eight patients (patients 3–10) low TM/BKG ratios corresponded with positive flow cytometric Pgp expression and/or function. This absence of ^{99m}Tc-sestamibi activity cannot be explained by tumour hypoperfusion or decreased cellular membrane potential since on the one hand imaging studies and histopathological examination revealed adequate perfusion of the primary tumours, and on the other hand the in vitro flow cytometric accumulation of the fluorescent cationic dye Rho 123 was not impaired [35].

Only one child (patient 1) presented with a discrepant result. In this patient, methodological, tumoral and vascular factors did not explain the discordance between flow cytometric and scintigraphic findings, since the primary tumour was well within the system resolution and not necrotic. Moretti et al. [41] and Hendrikse et al. [42] have recently shown that ^{99m}Tc-sestamibi is also a substrate for MRP. MRP expression was evaluated immunocytochemically in our patients (Table 1). In only one child (patient 3), suffering from NB stage 4, was MRP staining found in tumour imprints. In all other patients, and also in NB cells in the bone marrow smears of patient 1, no MRP was detected. Since ^{99m}Tc-sestamibi imaging cannot discriminate between Pgp and MRP activity, we believe that in vitro assays evaluating Pgp presence or absence are a prerequisite in clinical studies in which in vivo ^{99m}Tc-sestamibi imaging is used as a parameter of Pgp-mediated MDR.

Our data, which demonstrate the flow cytometric absence of Pgp in two stage 4 NB patients (patients 1 and 2) and the highest levels of expression in stage 1 NB (patient 6), GNB (patient 8) and GN (patient 9), also support the findings of Nakagawara et al. [15] and Bates

et al. [14]. These authors, using RNA detection assays, described increased Pgp expression in localised, more differentiated tumours. Ramani et al. [43], who applied non-radioactive in situ hybridisation and sensitive immunocytochemical techniques, also found statistically significant correlations between MDR1, Pgp expression and differentiation. In the latter study, Pgp immunoreactivity was correlated with favourable clinical variables such as age less than 1 year at diagnosis and stage 1, 2 and 4 S.

We also observed in four children (patients 3, 6, 8 and 9) dissociations between the flow cytometric expression level (MFI) and functionality value (Rho 123 retention ratio). This is in accordance with Bates et al. [44], who described increased levels of Pgp expression in NB cell lines treated with differentiating agents, without concomitant decreased drug accumulation. This finding inherently compromises the value of assays looking solely at Pgp expression and favours the use of techniques detecting Pgp functionality, such as ^{99m}Tc-sestamibi scintigraphy.

Finally, concern was raised by Piwnica-Worms et al. [20] that high levels of initial tracer uptake in liver, kidney and bowel, which probably reflect a normal transport function of Pgp, would require the judicious use of single-photon emission tomography to visualise abdominal tumours. In the present series, however, abdominal physiological activity did not interfere with image analysis and interpretation on planar imaging.

In conclusion: The findings presented suggest that ^{99m}Tc-sestamibi activity is not generally seen in newly diagnosed neural crest tumours without MYCN amplification, and that these imaging results might correlate with the presence of functional Pgp.

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European Journal of Nuclear Medicine Vol. 26, No. 4, April 1999

IMAGING WITH TECHNETIUM-99M SESTAMIBI DOES NOT PREDICT DRUG RESISTANCE OR OUTCOME IN CHILDHOOD NEUROBLASTIC TUMOURS

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European Journal of Nuclear Medicine, *submitted*

Imaging with technetium-99m sestamibi does not predict drug resistance or outcome in childhood neuroblastic tumours

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ABSTRACT

Purpose: Technetium-99m sestamibi (MIBI), a radiopharmaceutical used in tumor detection, is transported by the multidrug resistance proteins P-glycoprotein (P-gp) and multidrug resistance-associated protein (MRP1). Hence, imaging studies using MIBI are attractive to evaluate in vivo P-gp and MRP1 transport activities, and to predict drug resistance and outcome.
We evaluated MIBI imaging in children suffering from a neuroblastic tumor. These imaging results were compared with the P-gp and MRP1 status in vitro and the clinical outcome.
Patients and methods: Twenty children (mean age: 39.5 months, median follow-up time 38 months) with the diagnosis of neuroblastoma (n=14), ganglioneuro-blastoma (n=1) and ganglioneuroma (n=5) were evaluated by MIBI imaging at initial presentation. Scintigraphy was performed 20-30 minutes and 3.5-4 hours after injection of 740 Mbq/1.73 m^2 MIBI. P-gp expression and activity were determined immunocytochemically and/or flow cytometrically. For MRP1, an immunocyto-chemical assay was used.
Results: Sixteen tumors were MIBI negative, which suggests the presence of P-gp or MRP1. This was confirmed in 13/15 patients tested in vitro. However, 12/16 MIBI negative patients are alive without evidence of disease. Two patients were MIBI positive. This correlated with the absence of P-gp but not with the adverse clinical outcome of one of the patients. Two MIBI scans could not be interpreted because of overlapping physiological tracer uptake.
Conclusions: MIBI scanning in neuroblastoma is not useful in tumor detection. Absence of uptake correlates with the presence of MDR proteins and may reflect tumor differentiation. MIBI imaging does not offer useful information about clinical drug resistance in neuroblastoma.

INTRODUCTION

Neuroblastic tumors account for 8 % to 10 % of all malignancies in children. The variations in tumor locations and degrees of histopathological differentiation result in a spectrum of tumors, demonstrating diverse clinical and biological characteristics and behaviour[1]. Despite extensive research, the outcome for children older than one year with metastatic disease remains poor. Unfortunately, this is the case for approximately 40 % of the patient population with neuroblastoma. For these patients, the 3-year event-free survival rate does not exceed 30 % with current treatment protocols[2,3]. If unfavorable tumor characteristics are present, prognosis is even worse[1].
These high-risk patients are mainly treated with intensive multichemotherapy. A substantial number of them contract drug-resistant disease. The clinical resistance progressively increases with the intensity of the chemotherapy and is the highest at relapse after bone marrow transplantation[3,4].
In the past, the expression of the multidrug resistance (MDR) proteins P-glycoprotein (P-gp) and the multidrug resistance-associated protein MRP1 has been examined in neuroblastoma by several authors. These MDR proteins are adenosine triphosphate (ATP)-binding cassette (ABC) transporters, capable of transporting drugs against a drug concentration gradient and hence reducing the intracellular accumulation and

promoting cell survival[5,6]. P-gp acts on different clinically important drugs such as vinca alkaloids, anthracyclines and epipodophyllotoxins. MRP1, an organic anion transporter, causes resistance to vinca alkaloids and anthracyclines by transporting these compounds in complexes with glutathione. The enhanced expression of P-gp or MRP1 on the cell membrane of tumor cells, at diagnosis or induced by chemotherapy, was correlated with adverse clinical outcome in several human malignancies[7].

Although P-gp is detectable and functional in neuroblastoma cells[8-16], its clinical significance remains controversial. In contrast, the expression of the MRP1 gene has been shown to be associated with an increased relapse rate and reduced survival[17].

At first, we studied the expression of P-gp in neuroblastoma by an immunocytochemical alkaline phosphatase (APAAP) assay[12]. As functional assays might be more relevant, we used a flow cytometric test to quantify the transport capacity of P-gp in neuroblastoma cells in the presence or absence of MDR reversal agents. We observed a discordance between P-gp expression and activity in flow cytometric tests. Low P-gp activity but strong expressions were found in histologically well-differentiated tumors. On the contrary, high activity levels and weak expressions were seen in stage 4 neuroblastoma patients[16].

Technetium-99m (Tc-99m) sestamibi is a small lipophilic and cationic radiopharmaceutical. It enters the cell passively by diffusion, driven by negative transmembrane potentials, and is sequestered mainly in the mitochondria. Hence, it accumulates preferably in tissues characterized by high mitochondrial levels and consequently an increased amount of negative mitochondrial membrane potentials such as malignant tumors and the myocardium[18]. At present, Tc-99m sestamibi is used for myocardial perfusion studies and diagnostic imaging of a variety of tumors. The accumulation and retention of Tc-99m sestamibi is also influenced by extrusion mediated by P-gp or MRP1[19,20]. Hence, Tc-99m sestamibi imaging studies have become an attractive means to evaluate the in vivo transport activity of P-gp or MRP1, and to predict drug resistance and therapeutic outcome[21]. Consequently, Tc-99m sestamibi scintigraphy is being promoted as a non-invasive examination of drug resistance in several tumors, including neuroblastoma[22-26].

Previously, we investigated the usefulness of Tc-99m sestamibi scintigraphy in the screening of neural crest tumors for the presence of P-gp, and we found a strong correlation between the imaging results and the presence of functional P-gp[27].

In the present study, we evaluate the value of Tc-99m sestamibi imaging at initial diagnosis in 20 patients with neuroblastoma or ganglioneuroma. The imaging results were compared with the in vitro determination of P-gp and MRP1 and also with the clinical outcome of the patients.

PATIENTS AND METHODS

Patient and tumor characteristics:

Between February 1997 and September 2000, 20 children (mean age: 39.5 months, range 0-183 months) with the diagnosis of neural crest tumor were studied. The study was approved by the local Ethical Committee and informed consent was obtained from all parents.

Clinical data are listed in Table 1. All patients underwent computed tomography (CT)/magnetic resonance imaging (MRI) scanning of the primary tumor sites as well as metaiodobenzylguanidine (MIBG) and Tc-99m sestamibi scintigraphy at initial presentation. MIBG scintigraphy was performed as described previously[27]. Staging was done according to the International Neuroblastoma Staging System (INSS)[28]. Within two weeks after admission to the hospital, biopsy or resection of the primary tumor was performed to confirm the diagnosis histologically and to obtain samples for determination of biological parameters such as MYCN amplification, chromosome 1p-deletion and DNA content. Subsequently, if enough fresh tumor material was left, P-gp and MRP1 expression and/or function were determined.

Treatment was given according to current European protocols. Nine patients with low-stage neuroblastoma, ganglioneuroblastoma or ganglioneuroma (patients 12-20) were treated with surgery alone except for patient 19. The latter received one course of chemotherapy because the tumor was not resectable. After one course of chemotherapy, the tumor was completely removed by surgery and ganglioneuroma was the final histological diagnosis.

Table 1:Patient and tumor characteristics :

Patient[a]:	Histology[b]	Site[c]	Stage[d]	MIBG[e]	MYCNf	1p [g]	FISH/DI[h]	Therapy[i]	Resp. chemo	Outcome[j]/Follow up (mo)
1/B/35	NB	Adr	4	+	-	-	di/1	ch	yes	relapse, dead/34
2/B/11	NB	Adr	4	+	-	-	di/1	ch, surg	yes	alive NED/46
3/B/46	NB	Adr	4	+	+	+	0.92	ch, surg,abmt	yes	relapse, dead/9
4/G/54	NB	Bone	4 Rel	+	failure	failure	failure	ch, MIBG	yes	relapse, dead/20
5/G/15	NB	Abd	4	+	-	-	tri/1.53	ch, surg	yes	alive NED/40
6/G/1	NB	Adr	4S	+	-	-	tri/1.6	ch, expect	yes	alive NED/22
7/G/0	NB	Cerv	3	-	-	-	di/1.11	ch, surg	yes	relapse, dead/7
8/B/17	NB	Abd	3	+	-	14%	1.33	ch, surg	yes	relapse, dead/7
9/G/7	NB	DB	3	+	-	-	tri/1.68	surg, ch	yes	alive, NED/16
10/B/7	NB	Thor	3	+	-	-	2.2	ch, surg	yes	alive NED/25
11/G/12	NB	Abd	3	+	-	-	tri/1	ch,surg	yes	alive NED/48
12/B/3	NB	Adr	1	+	-	-	tri/1.19	surg		alive NED /43
13/G/5	NB	Thor	1	+	-	-	1	surg		alive NED /36
14/G/16	NB	Abd	1	+	-	-	tri/1	surg		alive NED /57
15/G/42	GNB	Abd	1	-	-	-	di/1	surg		alive NED /43
16/G/56	GN	DB		+	-	-	1.13	surg		alive NED /43
17/B/183	GN	Thor		-	-	-	di/1	surg		alive NED /41
18/B/137	GN	Thor		-	-	-	di/nd	surg		alive NED /38
19/B/34	GN	Thor		-	-	-	1	ch, surg	no	alive NED /38
20/G/109	GN	Abd		-	-	-	1	surg		alive NED /52

a : Patient number / B, Boys; G, Girls / age in months at diagnosis
b : Diagnosis : NB = neuroblastoma; GNB = ganglioneuroblastoma; GN = ganglioneuroma
c : Primary tumor localisation (Adr, adrenal; Abd, abdominal; Cerv, cervical; DB, dumbbell tumor)
d : INSS tumor stage; Rel, relapse
e : MIBG uptake in the primary tumor (and BM metastases in patients 1-5)
f : MYCN amplification: +, MYCN copy number >6; -, MYCN copy number < 6
g : 1p, allelic loss of chromosome 1p
h: DNA content determined by FISH/Flow cytometry: DI, DNA index; di, diploid; tri, triploid; nd, not determined
i: Therapy: ch, chemotherapy; surg, surgery; abmt, autologous stem cell transplantation; MIBG, MIBG therapy; expect, expectatio
j: Outcome: NED, no evidence of disease

According to the Infant Neuroblastoma Study (INES) protocol, one course of multidrug chemotherapy was given to an infant with stage 4S disease (patient 6) because of a positive Philadelphia score. Stage 3 patients received chemotherapy followed by second look operation according to the European Neuroblastoma Study Group (ENSG) protocol (patient 7, 8 and 11) or according to the INES protocol (patient 9 and 10). Stage 4 patients (patient 1 to 5) were given multichemotherapy comprising platinum derivatives, alkylating agents, and the P-gp substrates vinca alkaloids, anthracyclines and podophyllotoxins. Autologous stem cell transplantation was performed in one patient (patient 3). Treatment with retinoic acid was given for 6 months to patient 5. Patient 4 was treated for stage 4 disease as an infant. Five years after the end of therapy, she presented with bone pain and suffered from metastatic disease in the fibula and BM. Twenty months later, this child died from tumor progression.

Tc-99m sestamibi scintigraphy:
Patients were injected intravenously with 740 Mbq/1.73 m^2 Tc-99m sestamibi. Early (20-30 min after injection) and late (3.5-4 h after injection) guided spot and whole-body planar images were made using a single-headed Toshiba gamma camera (GCA 901 A) with a low-energy high-resolution collimator.

Image analysis:
Data were transferred to a Hermes processing system (Nuclear Diagnostics, Stockholm, Sweden) and tumor-to-background ratios (TM/BKG) were determined in addition to tumor and normal tissue percentage washout per hour (% W-O/h) as described previously[27]. Tc-99m sestamibi scans were interpreted by two nuclear physicians who were unacquainted with the patients' clinical history and P-gp/MRP1 status.

Sample collection:
In all patients a biopsy from the primary tumor was taken. Tumor imprints and samples disaggregated with collagenase (1000 U/ml for 1 hr at 37°C) were used for additional in vitro MDR tests (Table 2).
Bone marrow (BM) aspirations were performed routinely at diagnosis, and metastatic involvement was seen in five patients (patients 1-5). From all those patients, BM smears were available for immunocytochemical analysis. In addition, the BM of patient 1 was analyzed flow cytometrically. The BM of patient 2, obtained after one course of induction chemotherapy, contained 5 % neuroblasts and was also tested flow cytometrically.
Tumor cells of patients 2 and 11 could not be examined at initial diagnosis due to small biopsy samples. Cells were available for in vitro testing when the primary tumor was removed after multichemotherapy. In the case of patient 2, histological examination revealed a differentiation of the primary neuroblastoma into a ganglioneuroblastoma after six courses of chemotherapy.

In vitro determination of P-gp expression/activity and MRP1 expression:
Whenever possible, three different methods were applied to each tumor sample.
Immunocytochemical determination of P-gp expression was performed using the monoclonal antibodies 4E3 and MRK16 and an immunocytochemical APAAP technique[12]. MRP1 expression was determined using the monoclonal antibody MRPm6[27]. Samples were considered positive when a red staining of the cell membrane was found in at least one tumor cell.
For the flow cytometric determination of P-gp expression, a triple-color assay was applied, as described previously[16]. NB cells were reliably identified after electronic gating to include NB84-positive and CD45-negative cells. NB84 is a neuroblastoma marker and CD45 is a pan-leucocyte marker which is absent on NB cells. P-gp expression was considered positive if the mean fluorescence of the cells after labeling with the monoclonal antibody 4E3 or MRK16 was at least 10 % brighter compared to the fluorescence of the cells labeled with the isotype-matched control antibody[27].
Functional flow cytometric analysis was performed by studying the accumulation and retention of 200 ng/ml Rhodamine 123 (Rho123, Sigma, St. Louis, USA), a fluorescent substrate of P-gp, either in the absence or in the presence of 10µM verapamil (Isoptine®, Knoll, Ludwigshafen, Germany), a well-known P-gp inhibitor. The functional test was considered positive if the retention index (Rho123 retention in the presence of verapamil / Rho123 retention in the absence of verapamil) was at least 1.10 [27].
Samples of the chronic myeloid leukemia cell line K562 and its P-gp positive clone K562/VLB were simultaneously tested as negative and positive controls, respectively. In the MRP1 detection assays, the cell lines GLC4 and A549 were used as controls.

Table 2: Tumor-to-background ratios and determinations of P-gp

Patient No	In vitro sample	Tm/bkg ratio at diagnosis[c]	FCM Funct[d]	FCM[e] P-gp	APAAP[f] P-gp	APAAP[f] MRP1
1	tumor	1.0	np	np	np	np
	BM	1.2	-	-	-	-
2	tumor	**5.2**	np	np	np	np
	BM	**2.1**	np	np	-	np
	BM/ch		nm	-	np	np
	tumor/ch[a]		nm	+	np	np
3	tumor	0.9	+	+	-	+
	BM	1.0	np	np	-	+
4	Meta[b]	**1.8**	np	np	np	np
	BM	1.0	np	np	-	+
5	Tumor	1.1	+	+	-	+
	BM	1.2	np	np	-	-
6	Tumor	nl (1.57)	np	np	np	np
7	Tumor	1.0	+	+	-	-
8	Tumor	1.0	+	+	-	-
9	Tumor	nh (2.53)	+	+	+	+
10	Tumor	0.99	+	+/-	-	-
11	Tumor/ch[a]	1.10	nm	nm	-	np
12	Tumor	0.9	+	+	+	-
13	Tumor	1.07	+	+	-	-
14	Tumor	0.7	+	+	+	-
15	Tumor	1.2	+	+	-	-
16	Tumor	0.9	+	+	np	np
17	Tumor	1.01	+	+	np	np
18	Tumor	1.09	np	np	np	np
19	Tumor/ch[a]	0.94	+	+	+	-
20	Tumor	0.9	nm	+	np	np

a : ch: chemotherapy
b : Meta: metastasis
c : tumor-to-background ratio of the primary tumor (BM metastases in patient 1-3,5); nl, not measurable due to interference of liver tissue; nh, not measurable due to interference of the heart; **bold:** visible tracer captation
d : Result of flow cytometric functional test: -, negative; +, positive; np, not performed; nm, not measurable
e : Result of flow cytometric immunological test: +/-, 4E3 positive/MRK16 negative; np, not performed
f : Result of the immunocytochemical APAAP test: np, not performed

RESULTS

Tc-99m sestamibi imaging results:
Tumor (or BM) to background ratios (Table 2), as well as wash-out rates of the tumor (or BM) and the normal tissue were determined in each patient. Tracer accumulation was visualized on early and delayed images in two patients (patient 2 and 4). In patient 2, TM/BKG ratios in the primary tumor and in the occipital BM metastasis were 5.2 and 2.1, respectively. In patient 4, the bone metastasis in the fibula was clearly visible as illustrated in figure 1. The TM/BKG ratio of this lesion was 1.8. In this child, there was no tracer accumulation in the BM (TM/BKG ratio 1.0). The wash-out rates from the tumor were 14 % and 22 % for patients 2 and 4, respectively.

In 16 other primary tumors, the intratumoral Tc-99m sestamibi activity was comparable to the background activity (ranging from 0.7 to 1.2). In these patients, wash-out rates from the tumor were comparable to the wash-out rates from the surrounding normal tissue (data not shown).
In two patients (patients 6 and 9) the TM/BKG ratios were not measurable because the primary tumor was localized near sites of normal Tc-99m sestamibi physiological activity. Patient 6 with a neuroblastoma stage 4S had diffuse liver metastasis. Patient 9 had a dumbbell tumor located behind the heart.

P-gp and MRP1 status in two neuroblastoma patients with Tc-99m sestamibi accumulation:
The tumors of two patients (patient 2 and 4) that showed tracer accumulation were not tested in vitro at initial diagnosis because no tumor material was left. Smears of invaded BM were available for immunocytochemical analysis, and no P-gp expression was found. MRP1 was detected immunocytochemically in BM smears of patient 4.

The BM sample of patient 2, obtained after one course of chemotherapy, was tested flow cytometrically, and no P-gp expression was found. When the tumor of the same patient was analyzed after chemotherapy, P-gp expression was found using the flow cytometric assay.

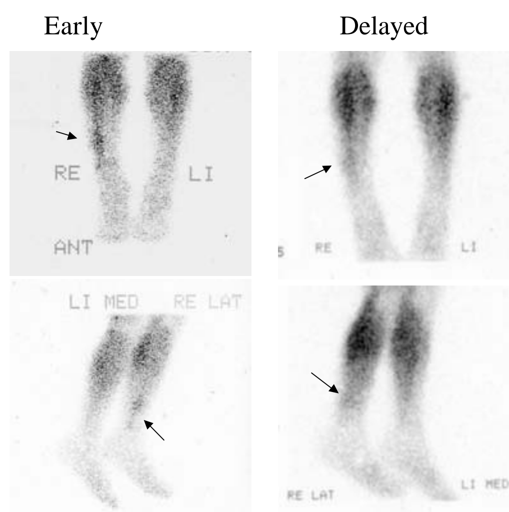

Figure 1: Bone metastasis located in the right fibula of a 4.5-year-old girl (patient 4). Tc-99m sestamibi uptake in the metastasis is observed on both early and delayed images (black arrows). The wash-out rate from the tumor was 22 %.

P-gp and MRP1 status in 16 patients without Tc-99m sestamibi accumulation:

In 13 out of 16 patients, P-gp activity was successfully determined. In 12/13 patients, a positive functional test result was found, which is concordant with the absence of tracer accumulation. One patient was not tested since no tumor material was available, and in 2/16 patients, the functional test was not interpretable. The flow cytometric immunological assay was successfully performed in 14/16 children, and a negative test result was obtained only for patient 1.

12/16 patients and 11/16 patients were successfully tested with the APAAP assay to determine P-gp and MRP1, respectively. P-gp was detected in only three patients, whereas the flow cytometric assays were positive in 13 patients. MRP1 was found immunocytochemically in two patients (patients 3 and 5).

Comparison of in vitro P-gp detection assays:

All samples tested with flow cytometric immunological and functional tests had concordant results: one patient was negative for both tests (patient 1); all other patients were positive in both assays.

P-gp expression was examined by flow cytometry and immunocytochemistry in 12 children, with concordance in five and disagreement in seven, flow cytometry always being positive for negative immunocytochemical test results.

Tc-99m sestamibi imaging results, P-gp/MRP1 status and clinical outcome:

The median follow-up of the 18 patients from whom Tc-99m sestamibi imaging results are interpretable is 39 months.

In 16 primary tumors, the intratumoral activity was comparable to the background activity, which might suggest the presence of P-gp or MRP1. Indeed, P-gp was found positive in 13 tumor samples that were flow cytometrically tested. Only three of these 13 patients had a bad outcome: a boy of 46 months old (patient 3) with stage 4 disease, MYCN amplification, 1p deletion and MRP1 expression; a boy of 17 months (patient 8) suffering from stage 3 neuroblastoma with 1p deletion; and a newborn with a cervical MIBG-negative neuroblastoma who developed resistant disease and died at the age of 7 months (patient 7). The other 10 P-gp positive patients, amongst them all the ganglioneuroblastoma and ganglioneuroma patients, are alive without any evidence of disease. None of these patients had MYCN amplification or 1p deletion (Table 1).

Although we did not find in patient 1 Tc-99m sestamibi uptake in the primary tumor and metastases, P-gp and MRP1 test results were negative. This boy, 35 months old at diagnosis, had a stage 4 neuroblastoma without MYCN amplification or 1p deletion. Initially, he responded well to chemotherapy, but he relapsed and died from resistant disease 34 months after diagnosis.

Tc-99m sestamibi enhancement was seen in the primary tumor and the BM metastases of patient 2, and the metastatic bone lesion of patient 4. There was no enhanced Tc-99m sestamibi uptake in the BM of the latter patient. In these patients, no tumor cells were left for in vitro determinations of P-gp or MRP1. BM smears of both patients were tested, and no P-gp expression was found immunocytochemically. MRP1 was positive in the BM of patient 4, which correlates with the imaging results.

Patient 4 was treated as an infant for neuroblastoma stage 4. At the age of 54 months she presented with BM and bone metastasis. After initial response, she developed resistant disease and died 20 months after the diagnosis of the relapse.

Forty-six months after initial diagnosis, patient 2 is alive without evidence of disease. Metastatic BM neuroblasts of this child did not express P-gp at diagnosis and after one course of chemotherapy. In contrast, P-gp was detectable in cells of the primary tumor after six courses of chemotherapy. At that moment, the tumor showed a histologically differentiated aspect (ganglioneuroblastoma).

DISCUSSION

In the present study, we evaluated Tc-99m sestamibi imaging in patients suffering from a neuroblastic tumor: neuroblastoma, ganglioneuroblastoma or ganglioneuroma. We compared these imaging results with the in vitro expression of the MDR proteins P-gp and MRP1 and with the clinical outcome of the patients.

Tc-99m sestamibi is currently being used as a tumor detection assay and, since it detects functional P-gp or MRP1, as an assay to examine drug resistance and to predict which patients might benefit from treatment with MDR modulators [21-26]. However, the data of our study show that in vitro confirmation of P-gp or MRP1 presence is an absolute prerequisite in clinical studies in which Tc-99m sestamibi imaging is used as a predictor of MDR mediated by P-gp or MRP1.

Tc-99m sestamibi scintigraphy has the following advantages. The technique can easily be performed. Patients can be evaluated at initial diagnosis and during or after chemotherapeutic treatment. Moreover, it is independent of biopsy or resection material. In our study, there was no suitable tumor material left for flow cytometric analysis in four patients, and in five others no tumor imprints were available. A potential limitation of Tc-99m sestamibi scanning is the clearance of Tc-99m sestamibi by the liver with excretion into the gallbladder and intestinal tract, resulting in high background activity in the abdomen. This activity can obscure tumor deposits in the proximity of the liver, as was the case in patient 6. The overlapping physiological uptake in the heart made the Tc-99m sestamibi imaging result of patient 9 uninterpretable. SPECT scintigraphy instead of planar images might resolve this problem.

In the majority of our patients (16/18), the tumors were not Tc-99m sestamibi-avid. Only two patients had tracer uptake in the tumor. Hence, Tc-99m sestamibi scanning is not useful as a tumor-seeking technique in neuroblastoma. This finding is confirmed by two other studies where stage 4 neuroblastoma patients were evaluated by Tc-99m sestamibi scintigraphy[25,26]. Özcan et al.[26] described a lack of Tc-99m sestamibi uptake in the primary tumor and metastatic foci of a 5-year-old boy with a neuroblastoma stage 4. Since the tumor of this patient was MIBG positive, the authors concluded that it was viable and hypothized that the lack of Tc-99m sestamibi uptake was related to the presence of P-gp.

Burak et al.[25] studied the primary tumors and metastases of 9 stage 4 neuroblastoma patients. None of the primary lesions and only 16 of 41 metastatic sites showed Tc-99m sestamibi accumulation.

In our study, the absence of Tc-99m sestamibi activity cannot be explained by tumor hypoperfusion, since imaging studies and histopathological examination revealed adequate perfusion of the primary tumors and the absence of necrosis. The size of the tumors was large enough to allow detection. An alternative explanation for non-visualization is the presence of pumps causing rapid efflux of the tracer.

In the present study, we found a correlation between the uptake (or the lack of uptake) of Tc-99m sestamibi in the tumor and the absence (or presence) of P-gp or MRP1 in vitro. These results are in accordance with previous study results[27].

Özcan et al.[26] suggested that the lack of Tc-99m sestamibi uptake was caused by the expression of P-gp, and hence predicted failure to respond to chemotherapy. However, P-gp was not determined in vitro, and the stage 4 neuroblastoma patient responded well to chemotherapy and remained disease-free.

Burak et al.[25] performed sequential Tc-99m sestamibi images during chemotherapeutic treatment and observed that all Tc-99m sestamibi-avid metastatic lesions became negative. In one group of patients, the Tc-99m sestamibi-negative lesions continued to demonstrate significant MIBG accumulation. These patients were clinically multidrug resistant. In the other group of “good-responders”, both Tc-99m sestamibi and MIBG scans were negative, suggesting resolution of the metastases. Based on these results, the authors concluded that serial Tc-99m sestamibi imaging identifies multidrug resistant stage 4 neuroblastoma patients. However, no in vitro determinations of P-gp were performed, nor was the histopathological aspect of the tumors after chemotherapy discussed.

Another shortcoming in both studies is the lack of prognostic biological information of the tumor cells.

Absence of Tc-99m sestamibi uptake caused by expression of P-gp or MRP1 in any tumor will have prognostic significance only if the expression in this particular tumor type is

correlated with a poor clinical outcome. In literature there is no consensus about the prognostic relevance of P-gp expression in neuroblastoma. Some authors found that enhanced P-gp expression was associated with poor prognosis[8,9]. Other studies confirm an inverse correlation of P-gp/MDR1 expression with MYCN oncogene amplification, an increased expression in localized, more differentiated tumors and an association with favorable clinical parameters and a good prognosis [10,11,14,15]. On the other hand, expression of MRP1 is considered to be associated with bad clinical outcome [17]. This is not confirmed in our study since two patients with MRP1 expression are doing fine. In the patients with unfavorable outcome, MRP1 is only sporadically detected. This might be caused by the low sensitivity of the APAAP assay in our setting.

Sixteen of our patients had no Tc-99m accumulation in the tumor. The majority of them had a concomitant P-gp positivity in vitro and were suffering from a low-stage neuroblastoma or a more differentiated tumor such as ganglioneuroblastoma or ganglioneuroma. In one stage 4 patient without Tc-99m accumulation at initial presentation, P-gp expression was found in the differentiated tumor after chemotherapeutic treatment. These findings suggest that P-gp might be a marker of differentiation in neuroblastoma.

In the past it was already hypothesized that an increase of MDR1 expression following drug treatment was due to chemotherapy-induced differentiation. Bates and colleagues[29] found an increase in MDR1 expression after treatment with retinoic acid, which also induces differentiation in neuroblastoma. The association between P-gp expression and differentiation in neural crest cells was also supported by the findings of Obana et al.[14], who analyzed fetal and adult adrenal glands. While P-gp expression could not be found in the adrenal medulla of the fetus, it was strongly present in those of the adult, and hence reflected a normal maturation process.

<u>Conclusion:</u>

Tc-99m sestamibi scanning in neuroblastoma is not useful in tumor detection or staging. Absence of radionuclide uptake at the tumor site does reflect the presence of functional P-gp but does not contribute to clinical drug resistance in neuroblastoma patients and hence does not give useful information concerning response to chemotherapy and outcome of the patients. Our findings strongly support the hypothesis that P-gp expression is a marker of differentiation in neural crest tumors.

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5 Discussie

Het neuroblastoom is een typisch voorbeeld van een tumor die (bij recidief) sterk chemotherapieresistent is. Het identificeren van prognostische parameters is onontbeerlijk om ook de overlevingskansen van de chemotherapieresistente neuroblastomen te verbeteren. In hoofdstuk 4 wordt geprobeerd een antwoord te vinden op de vraag of P-gp een parameter van slechte prognose is bij neuroblastomen.

In een eerste studie werden tumorstalen en beenmerguitstrijkjes van 23 neuroblastoompatiënten onderzocht met de immuuncytochemische APAAP techniek. Van zes kinderen werden zowel stalen bij diagnose als na chemotherapie of bij recidief onderzocht (Dhooge et al., 1997). In deze APAAP immuuncytochemische test werd er een extra incubatie met het substraat toegevoegd om de gevoeligheid van de test te verhogen. Favrot et al. (1991) en O'Meara et al. (1992) pasten immers immuuncytochemische technieken toe zonder signaalversterking en konden geen enkele positieve neuroblast in hun stalen terugvinden.
In onze studie werd géén prognostisch belang van P-gp expressie in neuroblastomen teruggevonden. In slechts 10 % van de stalen bij diagnose kon P-gp expressie aangetoond worden en dit enkel bij patiënten met niet-gelokaliseerde ziekte. De EFS en OS van deze positieve stadium 3 en 4 patiënten was echter niet verschillend van de negatieve patiënten. Er werd geen correlatie gevonden van P-gp positiviteit met andere prognostische parameters zoals *MYCN* amplificatie of chromosoom 1p deletie, en ook niet met histopathologische differentiatie. Evenmin werd inductie van P-gp expressie na chemotherapie gezien. Deze resultaten zijn sterk gelijklopend met de resultaten van Kurowski en Berthold (1998) die zich ook baseerden op een immuuncytochemische techniek met signaalversterking.
In vervolgstudies (De Moerloose et al., 1999a en 1999b) konden we echter aantonen door een vergelijking van de immuuncytochemische detectie met een flowcytometrische immunologische test dat de immuuncytochemische test in neuroblastoomstalen minder gevoelig is, dit in tegenstelling tot de resultaten die bekomen worden bij ALL patiënten.

Flowcytometrische immunologische testen zijn gevoelig en eenvoudig uit te voeren op stalen van hematologische maligniteiten. Vaste tumoren moeten echter gedissocieerd worden vooraleer ze flowcytometrisch onderzocht kunnen worden. In een tweede studie (De Moerloose et al., 1999b) gebruikten we 22 gedisaggregeerde stalen om een immunologische test en ook een functionele test toe te passen. Vier beenmergstalen met neuroblastoomcellen en zes neuroblastoomcellijnen werden eveneens getest.
Een vereiste voor de interpretatie van de flowcytometrische immunologische test was wel dat de neuroblasten duidelijk geïdentificeerd konden worden in de heterogene celsuspensie. Dit werd gerealiseerd door gebruik te maken van combinaties MoAbs, m.a.w. via multiparameter flowcytometrie. De neuroblastoomcellen werden geïdentificeerd aan de hand van positiviteit voor de neuroblastoommerker NB84 en negativiteit voor de leucocytenmerker CD45.
In deze flowcytometrische testen werd een opmerkelijke discrepantie vastgesteld tussen de expressie en de functie van P-gp. De sterkste P-gp expressie werd teruggevonden in de lagere neuroblastoomstadia en in de goed gedifferentieerde tumoren. De sterkste activiteiten en de zwakste expressie zagen we daarentegen in stadium 4 neuroblastomen.

Dit is in overeenstemming met het in vitro experiment van Bates et al. (1989) waarin een sterke P-gp expressie teruggevonden werd na de behandeling van een neuroblastoomcellijn met retinoïnezuur, zonder vermindering van de intracellulaire concentratie van P-gp substraten.

We pasten ook ^{99m}Tc sestamibi beeldvorming toe bij neuroblastoom- en ganglioneuroompatiënten als een in vivo functioneel onderzoek van P-gp. ^{99m}Tc sestamibi is een substraat van zowel P-gp als MRP1. De opname in maligne tumoren en efflux ervan kan een indirect bewijs leveren van de aanwezigheid van P-gp of MRP1.

Eerst bestudeerden we bij 10 kinderen ^{99m}Tc sestamibi beeldvorming bij diagnose van een neuroblastaire tumor en vergeleken we deze resultaten met in vitro bepalingen van P-gp (De Moerloose et al., 1999a). Er werd bij één patiënt opname van het isotoop gezien in de primaire tumor en de beenmergmetastasen. In vitro kon bij dit kind geen P-gp gedetecteerd worden. Negen andere patiënten hadden een negatieve ^{99m}Tc sestamibi scan en bij 8 van deze kinderen werd functioneel P-gp teruggevonden.

In een tweede studie (De Moerloose et al., *submitted*), werden 20 kinderen met een neuroblastoom, ganglioneuroblastoom en ganglioneuroom onderzocht met ^{99m}Tc sestamibi beeldvorming en immunologische en flowcytometrische testen voor P-gp. De doelstellingen van deze studie waren drievoudig. Enerzijds wilden we een idee krijgen over de detectiecapaciteit van primaire neuroblastomen en metastasen d.m.v. ^{99m}Tc sestamibi. Anderzijds bestudeerden we de correlatie met de in vitro P-gp status van de tumoren en met de klinische evolutie van de patiënten die gemiddeld 38 maanden werden opgevolgd.
Uit onze studie bleek dat ^{99m}Tc sestamibi beeldvorming bij neuroblastomen niet nuttig is als detectietechniek want slechts twee patiënten hadden opname in de primaire haard en metastasen. Er werd wel opnieuw een goede correlatie gezien met de in vitro P-gp bepalingen. Bij de twee kinderen met een positieve ^{99m}Tc sestamibi scan, beiden met een stadium 4 neuroblastoom, werd geen P-gp teruggevonden in vitro. Van de 16 andere, ^{99m}Tc sestamibi negatieve tumoren, konden er 15 in vitro getest worden en scoorden er 13 positief.
Anderzijds vonden we geen correlatie terug tussen het ^{99m}Tc sestamibi resultaat en de klinische evolutie van de patiënten. Onze resultaten toonden immers aan dat P-gp bij voorkeur teruggevonden wordt bij de prognostisch gunstige neuroblastomen (zoals de stadium 1 neuroblastomen, de ganglioneuroblastomen en de ganglioneuromen). Eén van de twee stadium 4 patiënten, die initieel P-gp negatief was en na 46 maanden nog steeds in remissie verkeert, vertoonde P-gp expressie in zijn primaire tumor bij resectie na 6 kuren chemotherapie. Histopathologisch werd toen eveneens een uitrijping van deze tumor naar ganglioneuroblastoom gezien.

De resultaten van onze studies over P-gp expressie en functie in neuroblastomen suggereren dat P-gp geassocieerd is met differentiatie en maturatie en géén kenmerk is van MDR. ^{99m}Tc sestamibi beeldvormingsonderzoek als indirecte indicator van P-gp aanwezigheid en chemotherapieresistentie zal bijgevolg geen nut hebben bij neuroblastaire tumoren.

ALGEMEEN BESLUIT EN TOEKOMSTPERSPECTIEVEN

MDR blijft een belangrijke uitdaging voor iedereen die met de behandeling van kanker geconfronteerd wordt. De rol van P-gp in het ontstaan van MDR wordt reeds meerdere jaren onderzocht. Nochtans blijven klinische studies waarin de prognostische relevantie van P-gp bij neuroblastomen en ALL op kinderleeftijd worden nagekeken, tegenstrijdige resultaten opleveren. Dit is hoofdzakelijk het gevolg van het gebruik van niet-gestandaardiseerde detectietechnieken.

Omdat P-gp een potentieel aangrijpingspunt is voor alternatieve behandelingen (ondermeer door het inschakelen van modulatoren), is het noodzakelijk om via betrouwbare en klinisch relevante testen de waarde van P-gp te bepalen.

In deze studie werd daarom eerst een grondige evaluatie gemaakt van enkele detectiemethoden van P-gp, nl. immuuncytochemie, flowcytometrische immunologische en functionele testen en een beeldvormingstechniek met het isotoop ^{99m}Tc sestamibi. De flowcytometrische technieken werden oppuntgesteld zodat, zowel voor leukemiestalen als neuroblastomen, multiparameter analyses konden toegepast worden.

Er werd een vergelijking gemaakt van immunologische en functionele testen bij kinderen met ALL. De gecombineerde immuuncytochemische en functionele P-gp positiviteit was een significante parameter voor een slechtere EFS. Deze bevindingen bevestigen dat sommige kinderen met ALL in aanmerking kunnen komen voor een behandeling met P-gp modulatoren. Dit zou in de toekomst geëvalueerd kunnen worden in klinische “trials”.

Sequentieel onderzoek tijdens de behandeling van twee patiënten met een lymfoblastencrisis van CML bevestigde de hypothese dat P-gp positieve resistente klonen ontstaan door een selectieproces tijdens de toediening van chemotherapie. We bevelen daarom aan dat P-gp niet enkel bij diagnose, maar op meerdere tijdstippen tijdens de behandeling geanalyseerd wordt bij patiënten met een onvolledig of afwezig antwoord op chemotherapie.

Bij neuroblastomen werd in ons onderzoek een dissociatie gevonden tussen de mate van P-gp expressie en P-gp activiteit. Geen van beide parameters stond in verband met klinische MDR. Beeldvorming met ^{99m}Tc sestamibi is niet bruikbaar in de detectie of staging van neuroblastomen. Evenmin kan op basis van deze beeldvormingsresultaten een voorspelling gemaakt worden over MDR en “outcome” van de neuroblastoompatiënten.

P-gp werd in onze studies teruggevonden bij de beter gedifferentieerde neuroblastaire tumoren zoals ganglioneuroblastomen en ganglioneuromen, en bij uitgerijpte tumoren na chemotherapie. Welke de exacte rol is van P-gp bij neuroblastaire tumoren is onbekend.

Klinische “trials” met P-gp modulatoren hebben geen zin bij hoog-risico neuroblastoompatiënten. VX 710 en difloxacine, producten die de activiteit van MRP1 in vitro inhiberen in neuroblastoomcellijnen, komen wel in aanmerking voor een evaluatie bij neuroblastoompatiënten met een resistente tumor.

Verder onderzoek, o.a. de evaluatie van andere drugresistentie eiwitten zoals BCRP, zal nuttig zijn om de multifactoriële resistentiepatronen bij bepaalde ALL en neuroblastoompatiënten te verklaren en om die patiënten een aangepaste en doeltreffende therapie te kunnen geven.

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