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LABORATORY INVESTIGATION

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BRIEF METHOD

MRP6 (ABCC6) Detection in Normal Human Tissues and Tumors

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MRP6 (ABCC6) is a member of the subfamily of the multidrug resistance proteins (MRPs, reviewed by Borst et al, 2000), but its putative role in multidrug resistance (MDR, reviewed by Moscow et al, 1997) is still under investigation. Closely related proteins such as MDR1 P-glycoprotein (P-gp, ABCB1, reviewed by Ambudkar et al, 1999), breast cancer resistance protein (BCRP, ABCG2; Doyle et al, 1998), and MRP1, -2, and -3 (ABCC1–3) are established MDR transporters. The exact range of substrates for MRP6 has not yet been determined, but a preliminary report suggested that MRP6 may be involved in the transport of certain anticancer agents, including anthracyclines and epipodophyllotoxins (M.G. Belinsky et al, Proceedings of the AACR, abstract 1510, 2001). Recently it was found that mutations in the *MRP6* gene cause pseudoxanthoma elasticum (PXE), an inheritable disorder of the connective tissue involving impaired visual acuity, skin lesions, and cardiovascular complications (Bergen et al, 2000). The expression of *MRP6* in normal human tissues has only been studied at the mRNA level. High *MRP6* mRNA levels were reported in liver and kidney, whereas low expression was found in a range of other tissues, including lung, intestines, retina, skin, and vessel walls (Bergen et al, 2000; Kool et al, 1999).

To study MRP6 at the protein level, three rat Mabs (M_6II-7, M_6II-21, and M_6II-31) were generated from rats immunized with a fusion protein containing amino acids 764 to 964 of human MRP6 (FP M_6II), according to described methods (Scheffer et al, 2000). Reactivity of these Mabs to full length MRP6 protein was shown in Western blots with fractions of *MRP6*-overexpressing HEK 293 cells. All Mabs reacted with the approximately Mr 180,000 MRP6 protein, that migrated slightly faster than the related MRP2 protein (ABCC2), as detected with the M_2III-6 Mab (Scheffer et al, 2000) in a control cell line (Fig. 1). Isotype specific secondary antibodies (Nordic, Tilburg, The Netherlands) showed that M_6II-7 and M_6II-31 were both of IgG2a subclass, whereas Mab M_6II-21 was of IgG1 subclass. Lack of cross reactivity of M_6II-7, M_6II-21, and M_6II-31 with MDR1 P-gp or MRP1, -2, -3, -4, and -5 family members, was concluded from staining results from cytospin preparations of several cell lines and Western blot experiments with protein fractions

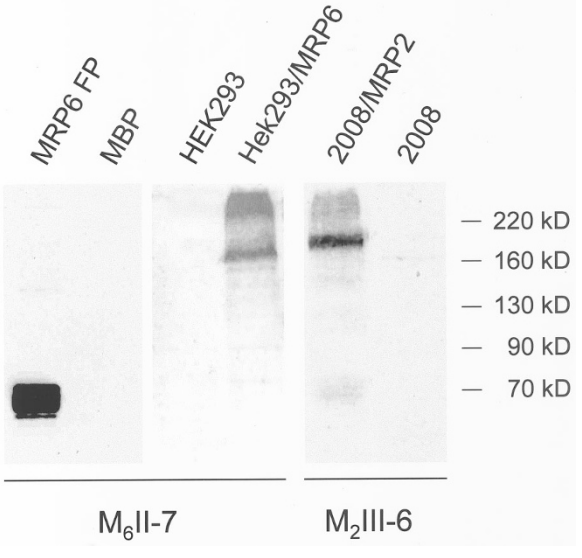

Figure 1.
Detection of the approximately 65 kD MRP6-MBP fusion protein and full length MRP6 from protein preparations of *MRP6*-transfected HEK 293 cells, using anti-MRP6 Mab M_6II-7. The approximately 180,000 kD MRP6 migrates slightly faster than MRP2, as shown by control lanes stained for MRP2, using M_2III-6 and *MRP2*-transfected cells. Total cell lysates were made as previously described (Scheffer et al, 2000). Ten to forty μg of cell lysates or fusion proteins were fractionated on a 7% polyacrylamide slab gel and transferred onto a nitrocellulose membrane by electroblotting. After blocking, the membrane was incubated for 2 hours with primary antibody in the appropriate dilution. Horseradish peroxidase (HRP)-labeled-anti-rat or -mouse serum (1:1000; Dako, Copenhagen, Denmark) was used as a secondary antibody. Enhanced chemoluminescence (Amersham, Buckinghamshire, United Kingdom) was used to detect Mab binding.

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Table 1. Reactivity of Mabs in Cell Lines

			Mab[d]								
Cell line	Origin	Transporter	JSB-1	MRPr1	M_2III-6	M_3II-9	M_4II-8	M_5I-1	M_6II-7	M_6II-21	M_6II-31
SW1573[a]	Lung		–	+/–	–	–	–	–	–	–	–
SW1573/2R160[a]		MDR1 P-gp	+++	+	–	–	–	–	–	–	–
GLC4[a]	Lung		–	+/–	–	–	–	–	–	–	–
GLC4/ADR[a]		MRP1	–	+++	–	–	–	–	–	–	–
HL60[a]	Leukemia		–	–	–	–	–	–	–	–	–
HL60/ADR[a]		MRP1	–	++	–	–	–	–	–	–	–
2008[a]	Ovarian		–	+	–	–	–	–	–	–	–
2008/MRP1[a]		MRP1	–	+++	–	–	–	–	–	–	–
2008/MRP2[a]		MRP2	–	+	+++	–	–	–	–	–	–
2008/MRP3[a]		MRP3	–	+	–	+++	–	–	–	–	–
Sf9/MRP4[b]	Insect	MRP4	–	–	–	–	+++	–	–	–	–
HEK293/MRP5[a]	Kidney	MRP5	–	–	–	–	–	+++	–	–	–
HEK293/MRP6[c]	Kidney	MRP6	nd	nd	nd	nd	nd	nd	+++	+++	++(+)

–, no reactivity; +/–, very weak reactivity; +, weak reactivity; ++, moderate reactivity; +++, strong reactivity; nd, not determined.

[a] (Scheffer et al, 2000).

[b] Spodoptera frugiperda Sf9 insect cells, transiently transfected with full length *MRP4* cDNA were a kind gift of Dr. R.A.M.H. van Aubel, University of Nijmegen, The Netherlands.

[c] Protein fractions of HEK293 cells transfected with full length *MRP6* cDNA were a kind gift of Dr. J. König, German Cancer Research Center, Heidelberg, Germany.

[d] (Scheffer et al, 2000) and unpublished.

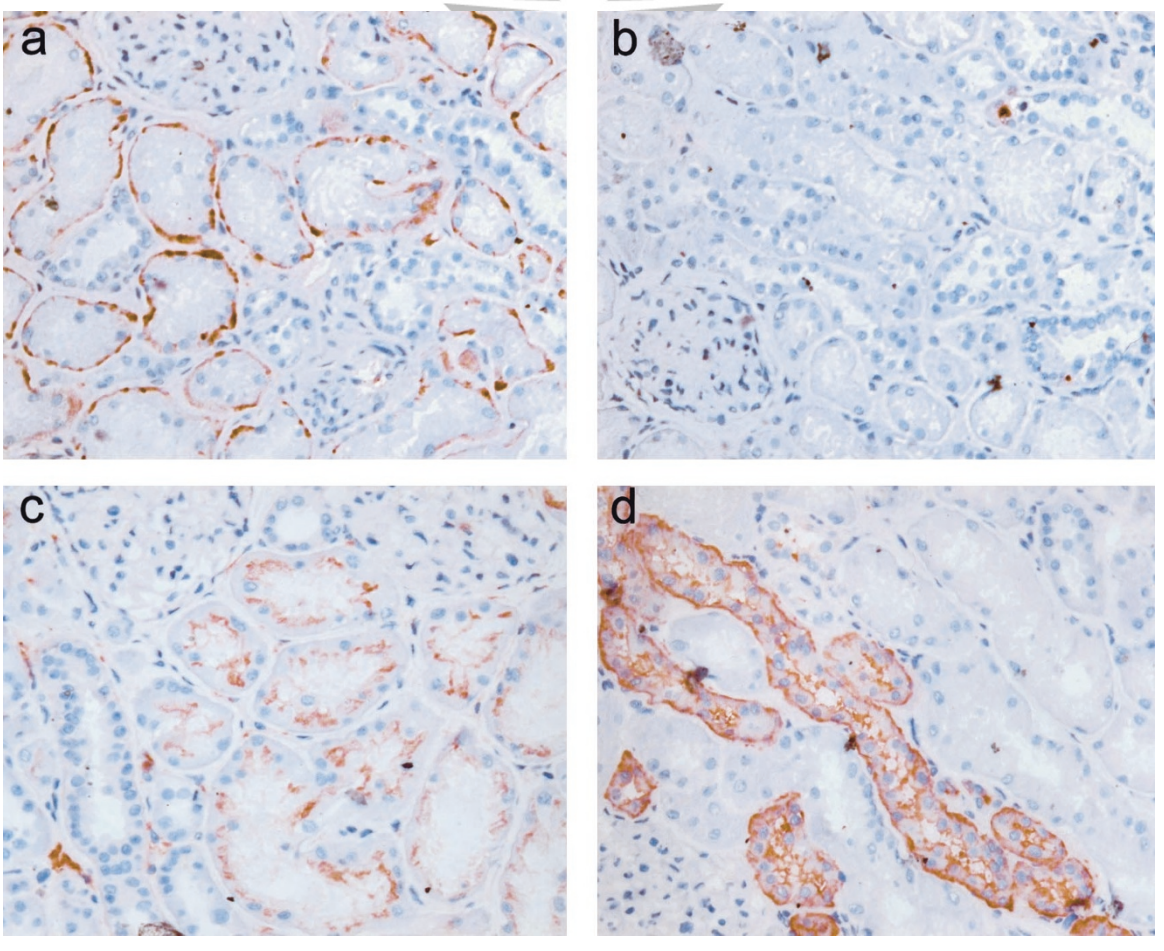

Figure 2.

Immunohistochemical staining of normal human kidney, using HRP-labeled rabbit-anti-rat serum, fluorescein isothiocyanate (FITC)-labeled tyramine, HRP-labeled rabbit $F(ab')_2$ -anti-FITC and aminoethyl carbazole as the chromogen. M_6II-31 shows the presence of MRP6 in the basolateral membranes of the proximal tubuli (a). Control stainings are with negative control antibody (b), anti-MRP2 mouse Mab M_2III-6 (c), and anti-Tamm-Horsfall rabbit polyclonal antiserum (d). M_2III-6 stains the apical membranes of the proximal tubules. The Tamm-Horsfall protein is localized in the early distal convoluted tubules and the thick ascending loops of Henle.

of these cell lines (Table 1). In line with normal tissue distribution studies at the mRNA level, considerable MRP6 staining in frozen sections of normal human tissues appeared to be restricted to kidney and liver. In the other tissues examined, including lung, brain, bladder, tonsil, spleen, heart, colon, adrenal gland, gall bladder, skeletal muscle, testis, ovary, placenta, parotis, esophagus, stomach, and pancreas, no MRP6 staining was observed. Surprisingly, also in sections of human skin and retina, no MRP6 staining could be observed, despite the fact that mutations in MRP6 were implicated in the connective tissue disorder PXE. Ongoing studies are investigating the direct or indirect role of (mutated) MRP6 in the eye and in skin lesions of PXE patients.

In kidney, MRP6 was located at the basolateral membranes of the proximal tubules (Fig. 2a). The staining pattern paralleled the staining observed with the anti-MRP2 Mab M_2III-6, but, as previously reported (Scheffer et al, 2000), the latter Mab rather stained the apical membranes (Fig. 2c). Control stainings with anti-Tamm-Horsfall polyclonal antiserum showed that this antigen does not co-localize with MRP6 (Fig. 2d). The Tamm-Horsfall protein is localized in the early distal convoluted tubules and the thick ascending loops of Henle.

In liver, the anti-MRP6 Mabs strongly stained the membranes of the hepatocytes (Fig. 3a). In contrast to data obtained with a polyclonal antiserum in rats (Madon et al, 2000), the staining appeared to be located exclusively at the basolateral membranes, leaving, at closer look, especially the cross-sections of the canalicular membranes as particularly unstained regions in a marked pattern. A control staining for MRP2 (known to be present at the canalicular membranes [Scheffer et al, 2000]) is shown in Figure 3c. To further investigate the presence or absence of MRP6 at the hepatocanalicular membranes, we performed a double staining for MRP6 and MRP2 using secondary reagents labeled with different fluorochromes. The proteins appeared to be present mutually exclusive at the basolateral and canalicular membranes, respec-

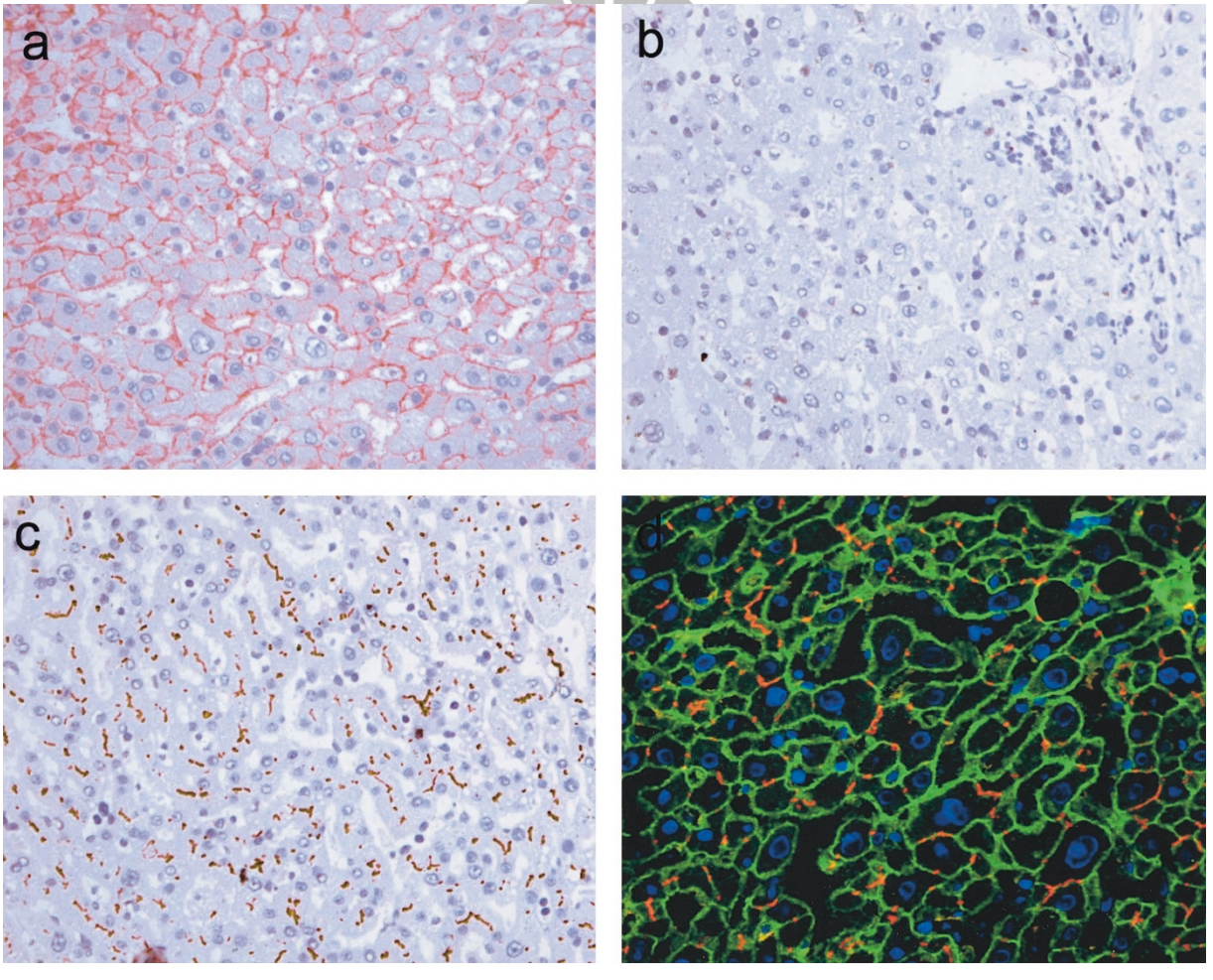

Figure 3.

Immunohistochemical staining of normal human liver, using HRP-labeled rabbit-anti-rat serum, FITC-labeled tyramine, HRP-labeled rabbit $F(ab')_2$ -anti-FITC and aminoethyl carbazole as the chromogen. M_6II-31 shows the presence of MRP6 in the basolateral membranes of the hepatocytes (a). Control stainings are with negative control antibody (b) and anti-MRP2 Mab M_2III-6 (c). M_2III-6 stains the canalicular membranes of the hepatocytes. d, A fluorescent double staining for MRP6 and MRP2 is shown, using rat anti-MRP6 Mab M_6II-31, HRP-labeled rabbit-anti-rat and tyramine-FITC (*green*), followed by staining with mouse anti-MRP2 Mab M_2III-6, biotin-labeled rabbit-anti-mouse and Cy3-labeled streptavidin (*red*). Nuclei were counter stained with 4',6-diamidino-2-phenylindole (DAPI; *blue*). MRP6 and MRP2 are present mutually exclusive at the basolateral- and canalicular membranes, respectively; no mixed fluorochrome color is observed.

tively, as shown by the absence of mixed fluorochrome color (Fig. 3d).

Examination of the reactivity of the anti-MRP6 Mabs on formalin fixed, paraffin embedded sections of human liver, using the citrate method as antigen retrieval, showed that the M_6II-7 and M_6II-21 Mab were unreactive on this type of material, whereas the M_6II-31 Mab was reactive, albeit less than observed in frozen sections.

In frozen sections of a small panel of human tumor samples from tissue of different histogenetic origins (including tumors of the intestine, stomach, testis, prostate, lung, pancreas, bladder, adrenal gland, cervix, neurologic tissue, mamma, ovary, kidney, and melanoma, $n = 34$), no clear MRP6 levels could be detected, indicating that the contribution of MRP6 to MDR in tumors may be rather limited.

The MRP6 specific rat Mabs should become valuable tools in further studying the substrate specificity and possible contribution of MRP6 to MDR and they should facilitate in-depth studies into the nature of the disease in PXE patients.

Acknowledgements

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References

Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I, and Gottesman MM (1999). Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Ann Rev Pharmacol Toxicol 39:361–398.

Bergen AA, Plomp AS, Schuurman EJ, Terry S, Breuning M, Dauwerse H, Swart J, Kool M, van Soest S, Baas F, ten Brink JB, and de Jong PT (2000). Mutations in ABCC6 cause pseudoxanthoma elasticum. Nat Genet 25:228–231.

Borst P, Evers R, Kool M, and Wijnholds J (2000). A family of drug transporters: The multidrug resistance-associated proteins. J Natl Cancer Inst 92:1295–1302.

Doyle LA, Yang WD, Abruzzo LV, Krogmann T, Gao YM, Rishi AK, and Ross DD (1998). A multidrug resistance transporter from human MCF-7 breast cancer cells [erratum in PNAS USA 1999; 96(5):2569]. Proc Natl Acad Sci USA 95:15665–15670.

Kool M, van der Linden M, de Haas M, Baas F, and Borst P (1999). Expression of human MRP6, a homologue of the multidrug resistance protein gene MRP1, in tissues and cancer cells. Cancer Res 59:175–182.

Madon J, Hagenbuch B, Landmann L, Meier PJ, and Stieger B (2000). Transport function and hepatocellular localization of mrp6 in rat liver. Mol Pharmacol 57:634–641.

Moscow JA, Schneider E, Ivy SP, and Cowan KH (1997). Multidrug resistance. Cancer Chemother Biol Response Modif 17:139–177.

Scheffer GL, Kool M, Heijn M, de Haas M, Pijnenborg ACLM, Wijnholds J, van Helvoort A, de Jong MC, Hooijberg JH, Mol CAAM, van der Linden M, de Vree JML, van der Valk P, Oude Elferink RPJ, Borst P, and Scheper RJ (2000). Specific detection of multidrug resistance proteins MRP1, MRP2, MRP3, MRP5 and MDR3 P-glycoprotein with a panel of monoclonal antibodies. Cancer Res 60:5269–5277.