

Expression of heat-shock protein 90 in glucocorticoid-sensitive and -resistant childhood acute lymphoblastic leukaemia

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Early reduction of leukaemic cells by chemotherapy is a strong predictor for treatment outcome in childhood acute lymphoblastic leukaemia (ALL). In ALL-(Berlin-Frankfurt-Münster) trials, early treatment response is assessed by the *in vivo* response to glucocorticoids (prednisone response, PR), the molecular background of which is unknown. The intracellular effects of glucocorticoids (GCs) are mediated by the glucocorticoid receptor (GR). In the absence of GC, the inactive GR resides within a multiprotein complex, consisting predominantly of the chaperone protein hsp90 (heat-shock protein 90). Until now, studies targeting GC resistance mainly focused on GR disorders and alterations of genes known to be associated with drug resistance. In addition, the GR multiprotein complex was associated with GC resistance in *in vitro* studies. We performed a case-control study for PR to investigate the association of *in vivo* GC resistance and hsp90 expression in childhood ALL. Hsp90 expression was assessed using a real-time PCR approach (Taqman technology) and Western blot technology. In this setting, we found no association of *in vivo* GC resistance and hsp90 expression. Therefore, we conclude that the expression of hsp90, the major component of the GR activating complex, is of minor importance for the *in vivo* GC resistance in childhood ALL.

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Introduction

Early reduction of leukaemic cell load by chemotherapy is one of the strongest predictive factors for treatment outcome in childhood acute lymphoblastic leukaemia (ALL).^{1,2} Within the Berlin-Frankfurt-Münster (BFM) group, the largest study group for the investigation of treatment in childhood ALL, the *in vivo* prednisone response (PR) has been the most important independent predictive factor for treatment outcome for more than 20 years.^{3–6} The PR is defined by the number of peripheral leukaemic blasts after 7 days of prednisone treatment and one intrathecal dose of methotrexate on day 1. It is, therefore, a clinical marker for the biological phenomenon of early leukaemic cell reduction. The threshold value for the distinction between good and poor PR is 1000 blasts/ μ l. In trial ALL-BFM 90, prednisone good responders (PGR) had a median 6-years event-free survival (EFS) of 82% in contrast to prednisone poor responders (PPR), who showed an EFS of 34%.⁶ In the 1990s, many studies confirmed the prognostic significance of early reduction of malignant cell load.^{7–10} Above all, prednisone has always been an important antileukaemic drug not only during the initial treatment phase but also during the intensified multichemotherapeutic protocol of ALL-BFM trial.

The biological background of the prednisone response has not yet been understood completely. Physiologically, the intracellular effects of glucocorticoids (GCs) are mediated by the human

glucocorticoid receptor (GR), a ligand-dependent transcription factor.¹¹ In the absence of GC, the inactive GR resides in the cytoplasm bound within a large multiprotein complex, the main protein of which is hsp90. GCs enter cells by passive diffusion and bind to the hormone-binding domain of the inactive GR, causing its change of conformation. Many proteins seem to function as chaperones and cochaperones during this process, for example, hsp90, hsp70, hsp40, p23 and Hop.¹² The conformational change of the GR finally results in the dissociation from the associated chaperone machinery followed by translocation of the receptor into the nucleus. There, homodimers bind to GC-responsive elements, which results in modulation of transcription of GC-responsive genes.¹³ Associated with the wide range of GC effects, a few hundred genes have been identified that are GC-regulated.¹⁴ In oncological disorders, GC function is mainly based on induction of cell death.^{15–17}

The GR has been the subject of intensive investigations, which aimed at identifying the cause of GC resistance. Studies with respect to receptor levels (sites/cell) in acute nonlymphoblastic leukaemia,¹⁸ distribution of GR splice variants in asthmatic patients,¹⁹ in chronic lymphocytic leukaemia²⁰ and in childhood ALL (own unpublished data, 2003), as well as studies in healthy subjects and childhood ALL patients investigating polymorphisms of genes known to be associated with drug resistance^{21–23} all attributed to approach the cause of GC resistance. Furthermore, studies targeting the protein heterocomplex involved in the activation of the GR showed an association with GC resistance in human leukaemic cell lines and mouse fibroblasts.^{24,25}

The current study aimed at investigating the association of PR and hsp90 RNA and protein expression in two matched case-control groups of childhood ALL patients.

Materials and methods

Patients and study design

We designed a case-control study for prednisone response to assess the association of glucocorticoid resistance and hsp90 expression in childhood ALL. Spare peripheral blood and bone marrow samples as well as clinical data from patients enrolled in trials ALL-BFM 90 and ALL-BFM 95 were used. Two different groups were closely matched for evaluating hsp90 RNA expression and protein expression. In both groups, cases were patients with poor *in vivo* PR ($n=10$ in the RNA group, $n=22$ in the protein group), controls were patients with good *in vivo* PR ($n=20$ in the RNA group, $n=22$ in the protein group). Matching criteria for both groups were initial white blood cell count ($<10\,000$, $10\,000$ to $<50\,000$ and $\geq 50\,000$ leukocytes/ μ l), sex, immunophenotype (T-ALL, non-T-ALL) and age at diagnosis (<1 year, 1 to <10 years and ≥ 10 years, Table 1).

Table 1 Distribution of clinicopathological features of childhood ALL patients, shown for both case-control groups

	RNA group		Protein group	
	Poor PR N=10 (100%)	Good PR n=20 (100%)	Poor PR n=22 (100%)	Good PR n=22 (100%)
Sex				
Male	6 (60.0)	12 (60.0)	15 (68.2)	15 (68.2)
Female	4 (40.0)	8 (40.0)	7 (31.8)	7 (31.8)
Age at diagnosis				
< 1 year	—	—	—	—
1 to <10 years	9 (90.0)	18 (90.0)	15 (68.2)	15 (68.2)
≥ 10 years	1 (10.0)	2 (10.0)	7 (31.8)	7 (31.8)
Initial WBC (/μl)				
< 10 000	—	—	—	—
10 000 to < 50 000	3 (30.0)	6 (30.0)	6 (27.3)	6 (27.3)
≥ 50 000	7 (70.0)	14 (70.0)	16 (72.7)	16 (72.7)
Immunophenotype				
Non-T-ALL	6 (60.0)	12 (60.0)	12 (54.5)	12 (54.5)
T-ALL	4 (40.0)	8 (40.0)	10 (45.5)	10 (45.5)

Cell samples

Spare bone marrow (BM) or peripheral blood samples taken from ALL patients at diagnosis before administration of GCs were used. Peripheral blood and BM samples had to have a minimum of 75% leukaemic blasts. All samples used were taken from patients of the ALL-BFM 90 and ALL-BFM 95 trials. Cells were collected after informed consent and according to the guidelines of the ethics committee. Peripheral blood mononuclear cells of five healthy donors were used as controls. Mononuclear cells of all samples were isolated using gradient centrifugation (Ficoll-Paque, Amersham Biosciences, Freiburg, Germany). Cells were cryopreserved and stored in liquid nitrogen in RPMI medium (Life Technologies, Inc., Karlsruhe, Germany) supplemented with 10% dimethyl sulphoxide (DMSO; Merck, Darmstadt, Germany) and 10% foetal calf serum (FCS; Life Technologies).

RNA extraction and cDNA synthesis

Total RNA was extracted from cells using the RNeasy kit (Qiagen, Hilden, Germany). RNAs were quantified photometrically. Prior to cDNA synthesis, RNA samples were DNase digested in order to avoid potential contamination with genomic DNA. cDNA was prepared by reverse transcription of total RNA using random hexamer oligonucleotides (MWG Biotech, Ebersberg, Germany) and Superscript II (Life Technologies).

Quantitative real-time RT-PCR analysis

For quantitative real-time RT-PCR analysis, the TaqMan technology (7700 Sequence Detector, Applied Biosystems, Foster City, CA, USA) was applied according to the manufacturer's instructions. Primers (MWG Biotech) and probes (Eurogentec Inc., Seriang, Belgium) were chosen using the Primer Express Software (Applied Biosystems). The following oligonucleotide was labelled with 5'-FAM (reporter) and 3'-TAMRA (quencher) and served as a probe: 5'-CCC TGC TGA GGA CTC CCA AGC GTA CT-3'. An hsp90-specific forward primer (Hsp90_ff: 5'-GCT TAT TTG GTT GCT GAG AAA GTA ACT-

3') was combined with an hsp90-specific reverse primer (Hsp90_rv: 5'-TTC CAC GAC CCA TAG GTT CAC-3'). Cycle conditions of the PCR were as follows: initial denaturation at 94°C for 10 min, 45 cycles of 94°C for 45 s–58°C for 30 s–72°C for 60 s and one final elongation step at 72°C for 8 min. Input amounts were optimized, resulting in threshold values between 20 and 35 cycles. Each sample was analysed in at least two independent assays with duplicate samples. Means of the gene of interest were normalized to GAPDH levels using GAPDH predeveloped Taqman assay reagents (PDAR; Applied Biosystems) according to the manufacturer's conditions. For specific PCR amplification of hsp90 or GAPDH the Taqman PCR Core Reagents (Eurogentec Inc.) were used according to the manufacturer's instructions. The relative expression levels were calculated using the comparative Ct method. Therefore, the target PCR Ct values are normalized to the Ct-value of the reference gene (GAPDH) by subtracting the GAPDH Ct-value from the target Ct-value. The relative expression level for each target PCR was calculated using the equation: relative expression = $2^{-(Ct(target)-Ct(GAPDH))} \times 100$.

Protein isolation

Protein from BM and peripheral blood mononuclear cells was isolated using a standard protocol for extraction of cytosolic and nuclear proteins.²⁶ Slight modification of the protocol included NP40 concentration (0.1% instead of 0.2%), omitting of AEBSF and an incubation period of 10–30 min after resuspension into Dignam buffer A. Protein concentration was measured photometrically directly before Western blotting.

Western blotting

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with two gels at a time. 40 μg of protein from total cell lysate was separated on 10% SDS gels for 90 min at 180 V (Owl Scientific Inc., Wobum, USA). Every gel was loaded with 40 μg protein from HeLa S₃ cells to normalize protein quantity. The gels were transferred to nitrocellulose membranes (Trans-Blot Transfer Medium, BioRad Laboratories,

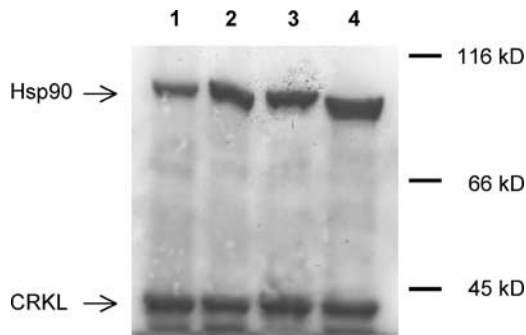


Figure 1 Western blot for hsp90 and CRKL in childhood ALL patients with good and poor *in vivo* response to GCs. Lanes 1–3 show hsp90 and CRKL expression in childhood ALL patients (relative hsp90 expression in lane 1: 0.57, in lane 2: 0.87, in lane 3: 0.81). Lane 4 represents hsp90 and CRKL expression in a HeLa cell line (expression arbitrary set to 1.0). The size of the proteins is indicated by a wide range colour marker (Sigma, Deisenhofen, Germany).

Richmond, USA) and blotted in parallel at 20V for 2 h. After blotting, gels (Commasie) and membranes (Ponceau S) were stained to document successful blotting. The membranes were incubated with a polyclonal anti-hsp90 antibody (sc-7947, SantaCruz Biotechnology, Santa Cruz, USA) at 4°C overnight. After washing (three times with PBS-T), gels were incubated with a goat anti-rabbit secondary antibody (sc-2030, SantaCruz Biotechnology) at 4°C overnight. Subsequently, the membranes were incubated with a polyclonal CRKL antibody (sc-9005, SantaCruz Biotechnology) at 4°C overnight. Washing and incubation with the secondary antibody was performed as described above. Every gel was loaded with HeLa S₃ cells as positive controls and with prestained markers (wide range colour marker, Sigma, Deisenhofen, Germany). Finally, the bands were visualized on a X-ray film (Cronex 5, AGFA, Mortsel, Belgium) using the electro-chemo-luminescence (ECL) method according to the manufacturer's instructions. Bands of the hsp90 protein appeared at approximately 90 kDa, bands of the CRKL protein at approximately 40 kDa (Figure 1). After scanning of the X-ray film, image analysis was performed on a Macintosh iMac computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image>). Linear detection of protein amounts was proven by hsp90 Western blot using a serial dilution of HeLa cell proteins.

Statistical analysis

Frequencies were calculated for descriptive purposes. Hsp90 RNA expression was assessed normalized to GAPDH. Hsp90 protein expression was calculated normalized to CRKL expression in HeLa S₃ cells. The correlation between median hsp90 RNA or protein expression and prednisone response was calculated by logistic regression analysis and a χ^2 test. Computations were performed using SAS software (SAS-PC Version 6.04, SAS Institute Inc., Cary, NC, USA).

Results and discussion

We performed a case–control study with closely matched childhood ALL patients to investigate the influence of hsp90

RNA and protein expression on the mechanism of *in vivo* GC resistance in childhood ALL.

Table 1 shows the distribution of the clinical features of all patients investigated in the two groups of our case–control study. Overall, the median expression of hsp90 was higher in ALL patients compared to peripheral blood mononuclear cells (PBMC) of healthy volunteers (Figures 2 and 3). This confirms data published by Yufu *et al*,²⁷ who found higher hsp90 protein expression in leukaemic cell lines as compared to PBMC. The reason for the upregulation of hsp90 in leukaemic cells has been suggested to be associated with cell differentiation and/or cell proliferation.²⁸ However, as PBMC do not reflect the optimal corresponding nonmalignant cell control for ALL blasts based on their different status of differentiation, these data should be confirmed with healthy donor lymphocyte precursor cells enriched for the corresponding differentiation status.

Comparing hsp90 RNA expression in leukaemia cells from patients with prednisone good and poor response, a slightly higher expression was found in PGR ($P>0.05$, Figure 2). Analysis of hsp90 protein expression (normalized to CRKL as a standard) revealed no major difference in expression levels between PGR and PPR (Figure 3). As in the RNA group, we

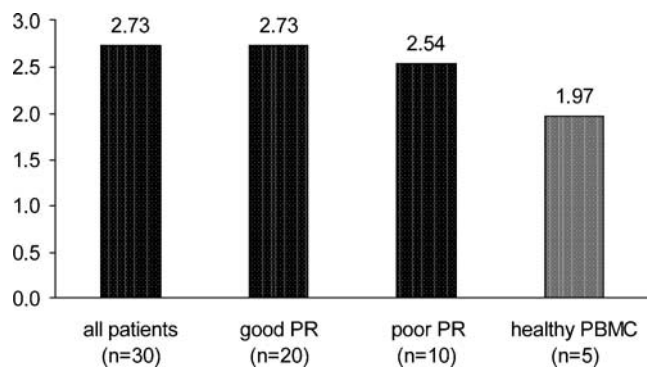


Figure 2 Relative median RNA expression of hsp90 in leukaemic blasts from childhood ALL patients. Results are shown for the total group of patients analysed ($n=30$), as well as for patients with good ($n=20$) and with poor ($n=10$) *in vivo* GC response. In addition, hsp90 RNA expression in PBMC of $n=5$ healthy donors is demonstrated.

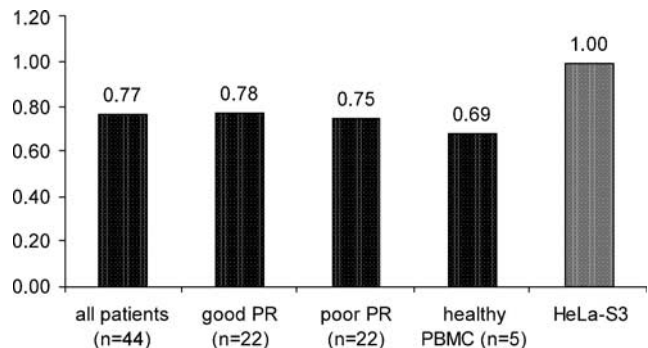


Figure 3 Relative median protein expression of hsp90 in leukaemic blasts from $n=44$ childhood ALL patients. Results are shown for the total group of patients analysed ($n=44$), as well as for patients with good ($n=22$) and with poor ($n=22$) *in vivo* GC response. In addition, hsp90 expression in PBMC of $n=5$ healthy donors and in HeLa S₃ cells is demonstrated. The hsp90 expression in HeLa S₃ cells was arbitrary set to 1.00.

Table 2 Median RNA and protein expression of hsp90 shown for the total group of patients and for the two prednisone responder groups

	Median Hsp90 RNA expression ^a (P ₂₅ -P ₇₅)	P	Median Hsp90 protein expression ^b (P ₂₅ -P ₇₅)	P
Total group	2.73 (1.47-3.83)		0.77 (0.58-0.93)	
Prednisone response				
Poor	2.54 (1.33-3.71)	>0.05	0.75 (0.56-0.92)	>0.05
Good	2.73 (1.50-3.97)		0.78 (0.63-0.98)	

^aNormalized to GAPDH.^bNormalized to CRKL expression in HeLa S₃ cells.**Table 3** Median RNA expression of hsp90 in subgroups of childhood acute lymphoblastic leukaemia patients

	N	Median hsp90 RNA expression ^a (P ₂₅ -P ₇₅)		P
		Poor PR	Good PR	
Sex				
Male	18	2.53 (1.49-3.67)	2.92 (1.54-4.42)	>0.05
Female	12	2.46 (1.33-6.74)	2.36 (1.33-3.29)	>0.05
Age				
1-9 years	27	2.79 (1.44-3.85)	2.31 (1.47-3.83)	>0.05
≥ 10 years ^b	3	1.32 (1.32-1.32)	3.60 (3.16-4.05)	>0.05
Initial WBC (/ μ l)				
10 000-49 999	9	1.33 (1.33-2.79)	3.03 (1.61-3.61)	>0.05
≥ 50 000	21	3.52 (1.55-4.12)	2.31 (1.47-4.04)	>0.05
Immunophenotype				
T-ALL	12	3.82 (1.87-6.88)	2.92 (1.53-3.98)	>0.05
Non-T-ALL	18	1.91 (1.33-2.99)	2.25 (1.50-3.85)	>0.05

^aNormalized to GAPDH.^bAge category ≥ 10 years comprises only one case and two controls.**Table 4** Median protein expression of hsp90 in subgroups of childhood acute lymphoblastic leukaemia patients

	N	Median hsp90 protein expression ^a (P ₂₅ -P ₇₅)		P
		Poor PR	Good PR	
Sex				
Male	30	0.77 (0.57-0.98)	0.84 (0.73-1.04)	>0.05
Female	14	0.59 (0.52-0.78)	0.65 (0.44-0.81)	>0.05
Age				
1-9 years	30	0.79 (0.59-0.98)	0.77 (0.63-0.96)	>0.05
≥ 10 years	14	0.52 (0.35-0.77)	0.84 (0.44-1.04)	>0.05
Initial WBC (/ μ l)				
10,000-49,999	12	0.58 (0.37-0.74)	0.80 (0.58-0.86)	>0.05
≥ 50,000	32	0.79 (0.58-0.97)	0.77 (0.63-1.04)	>0.05
Immunophenotype				
T-ALL	20	0.88 (0.73-0.98)	0.75 (0.59-0.98)	>0.05
Non-T-ALL	24	0.58 (0.42-0.76)	0.80 (0.64-1.01)	>0.05

^aNormalized to CRKL expression in HeLa S₃ cells.

observed a slightly higher hsp90 expression in PGR compared to PPR. Therefore, no statistically significant correlation between hsp90 expression and prednisone response was found in either groups investigated ($P > 0.05$ for RNA and protein expression, Table 2). Even subgroup analysis including known criteria for high risk in childhood ALL (high initial white blood cell count, T-cell immunophenotype, age ≥ 10 years and male sex) showed no statistically significant difference in hsp90 RNA and protein expression of the two responder groups (Tables 3 and 4).

Physiologically, GCs enter the cell by passive diffusion and bind to the glucocorticoid receptor. The functional GR resides in the cytoplasm bound within a multiprotein complex consisting of heat-shock chaperone and cochaperone molecules, necessary to activate hormone binding by the GR and to shuttle the GC-GR complex into the nucleus.²⁹⁻³¹ Miyata and Yahara³² showed that the hsp90-containing GR complex binds to filamentous actin *in vitro*, while the hsp90-free form of the receptor does not. This mechanism is thought to help anchoring the GR in the cytoplasm. Absence of hsp90 protein, the main chaperone within the respective multiprotein complex, was shown to be related to GC resistance in human lesion-derived cell lines.³³ Furthermore, expression of aberrant hsp90 protein was associated with GC resistance in leukaemic cell lines.²⁴

Our study excludes a differential expression of normal hsp90 RNA and protein in leukaemic blasts taken from *in vivo* GC-sensitive and -resistant childhood ALL patients at diagnosis. It, thereby, excludes a translational defect from hsp90 RNA to protein as well. Nevertheless, it does not completely exclude a putative influence of aberrant hsp90. In addition, the coex-

istence of a small fraction of GC-resistant cells among the total blast load, which may aberrantly express hsp90, can also not be excluded by this study.

This study used human primary leukaemic cells from trials ALL-BFM 90 and ALL-BFM 95. In ALL-BFM trials Bcr-Abl expression is a negative prognostic marker and is, therefore, investigated routinely. Bcr-Abl has been reported to be an hsp90-associated protein. In Bcr-Abl-positive K562 cells and in HL60 cells stably transfected with Bcr-Abl, geldanamycin (GA) caused a downregulation of Bcr-Abl and increased the sensitivity of Bcr-Abl-positive cells to doxorubicin.³⁴ Since GA exerts its function by binding to hsp90 and by degradation of hsp90-associated proteins such as Bcr-Abl, hsp90 expression itself could influence resistance to chemotherapy in Bcr-Abl-positive cells. In addition, CRKL, which we used as a standard for hsp90 protein expression, has been reported to be a central molecule within the Bcr-Abl signalling pathway.³⁵ Within the cases of this study investigated for hsp90 protein expression two patients with a Bcr-Abl translocation were found. Therefore, we re-evaluated the hsp90 protein expression of all patients within our case-control group excluding the two Bcr-Abl positive cases and their matched controls. The median hsp90 expression, though, remained unchanged. Therefore, the Bcr-Abl-positive patients were not excluded from the study.

To summarize, hsp90 expression was higher in leukaemia cells from patients with childhood acute lymphoblastic leukaemia compared to PMNC of healthy controls. However, we did not find a correlation between hsp90 expression and GC resistance in childhood ALL using quantitative real-time

RT-PCR and Western blot technologies in two closely matched case-control groups.

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