Regulation of mRNA Expression in Drug-sensitive and Drug-resistant Gastric Carcinoma Cells Is Independent of YB-1 Expression

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Abstract. *Y-Box protein 1 (YB-1) is a multifunctional cellular protein expressed in a range of mammalian cells, including human cancer cells. It is involved in the regulation of various genes including cancer-associated genes, but the full range of target genes and regulatory mechanisms have not been fully elucidated. To identify global mRNA expression patterns that are potentially regulated by YB-1, a previously established and well-characterized cell model derived from drug-sensitive (EPG85-257P/tetR/YB-1) and multidrug-resistant (EPG85-257RDB/tetR/YB-1) gastric carcinoma cells in which the expression of YB-1 can be inhibited by tetracycline-dependent activation of the RNA interference (RNAi) pathway, was analyzed by microarray technology. By this approach, various potentially regulated genes encoding members of important cellular pathways such as the Jak/STAT, VEGF and the MAP-kinase signaling pathways were identified. Independent validation of these findings by quantitative real-time reverse transcriptase polymerase chain reaction and Western blot did not confirm these regulatory effects. In conclusion, the findings suggest that YB-1 is not directly involved in the regulation of mRNA expression in drug-sensitive or drug-resistant gastric carcinoma cells.*

The Y-box is an inverted version of the classical CCAAT-box, a genetic region which is a component of many promoters in eukaryotic genes. Y-box binding proteins are a highly conserved class of proteins which have been implicated in the regulation of transcription and translation, but their exact biological functions are still poorly understood (1, 2). One of the best characterized proteins from this family is the Y-Box protein 1 (YB-1). It is a 42 kDa protein consisting of 322 amino acid residues. The approximately 19 kb spanning YB-1 encoding gene contains 8 exons and is localized in the chromosomal region 1p34. Originally, YB-1 was identified as a factor which binds to the Y-box in the promoter region of human major histocompability class II complex genes (3).

Y-box proteins including YB-1, belong to the family of cold-shock domain (CSD) proteins. The CSD of YB-1 shows 43% homology to that of bacteria, but YB-1 has not been demonstrated to be involved in cold-shock response. YB-1 contains three different domains: (i) an *N*-terminal domain (A/P domain) commonly variable in CSD proteins, (ii) a highly conserved CSD, and (iii) a C-terminal domain (CTD). The A/P domain plays a role in the regulation of transcription (1). The CSD can bind to single-and double-stranded nucleic acids, including oligonucleotides as well as DNA and RNA. It consists of two conserved ribonucleoprotein particles (RNP) motifs involved in intracellular transport processes and in the control of translation. The CTD contains alternate acidic and alkaline amino acid residues (B/A repeats) which play a role in protein-protein interactions. These B/A repeats are a common feature of many proteins which are translocalized between the cytoplasm and the nucleus following interaction with ribonucleoprotein complexes (4). Furthermore, two regions were identified within the CTD which are involved in the regulation of the subcellular distribution of YB-1: (i) the noncanonical nuclear localization signal (NLS), and (ii) the cytoplasmic retention site (CRS) (5).

Although YB-1 has been found to be expressed in many neoplastic tissues, it is unknown whether or not it plays a causative role in the development of cancer (2). *In vivo* investigations using a YB-1 over expressing transgenic mouse model demonstrated that YB-1 is involved in the development of invasive breast cancer (6). In studies analyzing expression and subcellular localization of YB-1 in tissue samples prepared from cancer patients, YB-1 was found to play a role in the regulation of the expression of oncogenes, *e.g.* in overexpression of Her-2 in breast cancer (7). Similarly, other cancer-related proteins, such as the

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Key Words: YB-1, gene regulation, gene expression, RNA interference.

multidrug resistance (MDR)-associated drug transporter MDR1/P-glycoprotein (MDR1/P-gp), can be regulated by YB-1 (8). In these tumors, YB-1 was identified as an independent prognostic and predictive marker (7, 9).

In spite of these observations, it is not clear whether the expression of YB-1 is directly associated with several differentially expressed genes in human cancer cells. In the present study, to analyze a potential direct role of YB-1 in regulation of gene expression in cancer cells, the global gene expression pattern in the presence and absence of YB-1 were analyzed in previously established and well-characterized cell models (10). In these cell lines, which were derived from the human gastric carcinoma cell line EPG85-257P (11) and its multidrug-resistant subline EPG85-257RDB (12), the expression of YB-1 can be downregulated by tetracycline exposure. Treatment with tetracycline induces the expression of short hairpin RNAs (shRNAs) directed against the YB-1-specific mRNA. The shRNAs cause a silencing of YB-1 expression *via* the RNA interference (RNAi) pathway.

Materials and Methods

Cell culture and anti YB-1 shRNA expressing cancer cell lines. Human carcinoma cells were grown in Leibovitz L-15 medium (Biowhittaker, Walkersville, MD) supplemented by 10% fetal calf serum (FCS) (GIBCO/BRL, Grand Island, NY), 1 mM L-glutamine, 6.25 mg/l fetuin, 80 IE/l insulin, 2.5 mg/ml transferrin, 0.5 g/l glucose, 1.1 g/l $NaHCO_3$, and 1% minimal essential vitamins in a humidified atmosphere of 5% CO_2 at 37˚C. Establishment and characterization of the tetracycline-inducible anti-YB-1 shRNA expressing cell variants EPG85-257P/tetR/YB-1 and EPG85-257RDB/tetR/YB-1 was described in detail previously (10). Anti-YB-1 shRNA-expressing cell lines were derived from the human gastric carcinoma cell line EPG85-257P (11) and its multidrug-resistant, MDR1/P-gp-positive derivative EPG85-257RDB which was established by *in vitro* exposure to daunorubicin (Daunoblastin®; Pfizer Pharma GmbH, Berlin, Germany) (12). These cancer cells are stably transfected with the tetracycline repressor encoding expression vector pcDNA6/TR (Invitrogen, Carlsbad, CA, USA) and the expression vector pTER (13) containing a modified RNA Polymerase III-dependent H1-RNA promoter with a tetracycline operator region driving the expression of a highly effective shRNA directed against the YB-1-specific mRNA. In cells engineered to express the bacterial tetracycline repressor, anti-YB-1 shRNAs are not synthesized in absence of tetracycline. For maintenance of tetracycline repressor expression and inducible anti-YB-1 shRNA expression, the medium was supplemented with 10 μg/ml Blasticidin S (Invitrogen) and 400 μg/ml Zeocin (Invitrogen); 2 μg/ml tetracycline (doxycycline, Invitrogen) were used for induction of anti-YB1 shRNA expression.

Northern blot. For confirmation of RNAi-dependent silencing of YB-1 mRNA expression, Northern blot analysis was performed as described previously (10). Blots were hybridized with 25 ng of a YB-1 homologous cDNA fragment labeled with [^{32}P] dCTP by random primed labeling (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). As a control for equal RNA loading the membranes were stripped and rehybridized with a fructose-bisphosphate aldolase-specific cDNA probe.

Western blot. Western blotting was performed for confirmation of RNAi-dependent down-regulation of YB-1 protein and for validation of global gene expression analyses as described previously (10). YB-1-specific band was detected using polyclonal rabbit antibodies (Cell Signaling Technology Inc., Danvers, MA, USA) at 1:1,000. Potential differentially regulated factors were detected using polyclonal rabbit antibodies against MAP3K14 (NIK) (Cell Signaling Technology;1:1,000), cPLA2 (Cell Signaling, Technology, Inc.;1:1,000), and Akt2 (Chemicon International, Inc.; 1:1,000). As a loading control, mouse monoclonal antibody (mAb) directed against actin (mAb 1501R; Chemicon, Temecula, CA, USA) was used at 1:5,000. Primary antibody incubation overnight at 4˚C was followed by incubation with HRP peroxidase-conjugated goat anti-rabbit IgG (1:2,000) (A-1949; Sigma, St. Louis, MO, USA) or goat anti-mouse IgG (1:10,000). Protein-antibody complexes were visualized by chemiluminescence developing reagents (ECL System, GE Healthcare Bio-Sciences Corp.).

RNA preparation. Total cellular RNA was isolated from 10^7 cells in logarithmic growth phase after 5 days tetracycline exposure or without any treatment, using the Qiagen RNeasy Mini Kit, following the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). RNA was quantified by UV spectroscopy and its quality was checked by analysis on a LabChip (BioAnalyzer, Agilent Technologies, Santa Clara, CA, USA). Samples were stored at –80˚C.

Array hybridization. For array hybridization, cDNA was synthesized from 5 μg total RNA, starting with annealing to 5 pmol/μl T7-(dT)24 primer (HPLC purified; MWGBiotech, Ebersberg, Germany) at 70˚C for 10 min. Reverse transcription, second-strand synthesis and cleanup of double-stranded cDNA were performed according to the protocols of the array manufacturer (Affymetrix, Santa Clara, CA, USA). Synthesis of biotin-labeled cRNA was performed using the BioArray High Yield RNA Transcription kit (Enzo Diagnostics, Farmingdale, NY, USA). cRNA concentration was determined by UV spectroscopy and the distribution of cRNA fragment sizes was checked by analyzing the samples on a LabChip (BioAnalyzer). Fragmented cRNA was hybridized to the HGU133 2.0 array (Affymetrix) at 45˚C in a hybridization oven for 16 h. Subsequent washing and staining of the arrays were performed using the GeneChip fluidics station protocol EukGE-WS2. Finally, probe arrays were scanned using the GeneChip System confocal scanner (Hewlett-Packard, Santa Clara, CA, USA).

Analysis of microarray data. Micorarray data were analyzed using the Gene Chip Operating Software 2.1 (GCOS 2.1; Affymetrix). First signal log ratios (SLR) were calculated by comparison of fluorescence intensities of tetracycline-treated to untreated samples, followed by fold change generation. All samples have been normalized by calculating the ratio of tetracycline repressor (tetR)-synthesizing cells (EPG85-257P/tetR, EPG85-257RDB/tetR) untreated to treated, as well as by calculating the ratios of tetR-expressing cells to double transfected cells expressing tetR as well as the anti-YB-1 shRNA (EPG85-257P/tetR/YB-1, EPG85-257RDB/tetR/YB-1), untreated to treated. A fold change limit of ±2 has been chosen as the criterion for differential expression.

Table I. *Oligodeoxynucleotide primers, sequences, annealing temperatures and PCR fragment lengths used for quantitative real time RT-PCR.*

Oligodeoxynucleotide primer	Sequence	Annealing temperature (˚C)	Size of PCR product (bp)
ALD fw	CAA TGT AAG GAA CGG ATA TGG	58	258
ALD rev	TTC CCC ACT CTC ACT ATT CTG		
AKT2 fw	ATG AAT GAG GTG TCT GTC ATC	57	214
AKT2 rev	TGC TTG AGG CTG TTG GCG ACC		
CHP fw	CGG ATC ATC AAT GCC TTC TT	56	152
CHP rev	AGT TTG TTG CTT CGG CTG TT		
IL6ST fw	CAG CAT GAA TCC AGT CCA GA	61	188
IL6ST rev	CCC TCA GTA CCT GGA CCA AA		
NFAT5 fw	CAA CAA GCT GCT TTC CAA CA	53	161
NFAT5 rev	ATG GTT CCT TGT TGG CTT TG		
MAP3K14 fw	CAA GCC TCT GAA GGA ACC AG	62	209
MAP3K14 rev	AGG GAT GAG GCA GTC TGC TA		
PIAS2 fw	AGA AAA AGC CCA CCT GGA TT	52	190
PIAS2 rev	CAC GGT TGG CTG GAT ACT TT		
SMAD2 fw	CGA AAT GCC ACG GTA GAA AT	53	223
SMAD2 rev	CCA GAA GAG CAG CAA ATT CC		
SRC fw	GGC TAC ATC CCC AGC AAC TA	55	173
SRC rev	TGA GAG GCA GTA GGC ACC TT		
TBP fw	TAT AAT CCC AAG CGG TTT GC	54	207
TBP rev	CCC CAC CAT GTT CTG AAT CT		
YB-1 fw	CAA TGT AAG GAA CGG ATA TGG	54	289
YB-1 rev	TTC CCC ACT CTC ACT ATT CTG		

fw, Forward; rev, reverse; ALD, fructose-bisphosphate aldolase; AKT2, v-akt murine thymoma viral oncogene homolog 2; CHP, calcium-binding protein P22; IL6ST, interleukin 6 signal transducer (gp130, oncostatin M receptor); NFAT5, nuclear factor of activated T-cells 5 (tonicity-responsive); MAP3K14, mitogen-activated protein kinase kinase kinase kinase 14 (nuclear factor-inducing kinase); PIAS2, protein inhibitor of activated STAT 2; SMAD2, SMAD family member 2; SRC, v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian); TBP, TATA box binding protein; YB-1, Y-box binding protein 1.

Quantitative real-time RT-PCR for detection of mRNA expression. To validate the results obtained by the Affymetrix HGU133 chips, quantitative real time reverse transcription polymerase chain reaction (qRT-PCR) was performed using a Light Cycler instrument and LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany) containing 500 nM of each gene specific oligodeoxynucleotide primer (Table I) and 2 mM $MgCl_2$. The following PCR program was performed to determine expression levels of the genes of interest: enzyme activation for 10 min at 95˚C was followed by 40 cycles of denaturation (15 s at 95˚C), annealing (5 s at target-specific temperatures, Table I), and elongation (15 s at 72˚C). Specificity was confirmed by melting curve analysis (0.5˚C/s from 65˚C to 95˚C). For relative mRNA quantification, the relative calibrator normalized quantification method with efficiency correction was applied, *i.e.* in each run, a calibrator consisting of cDNA prepared from EPG85-257RDB/tetR/YB-1 cells was measured in parallel to all other samples. For the calculation of fold change values, RelQant software (Roche Diagnostics) was used to generate normalized ratios. For normalization, TATA-binding protein (*TBP*) was used as a housekeeping gene. Normalized values were transformed into fold changes.

Results

RNAi-mediated down-regulation of YB-1 mRNA. Northern blot analysis (Figure 1) confirmed that tetracycline treatment of stable anti-YB-1 shRNA expression vector transfected cell lines resulted in down-regulation of YB-1-specific bands in both EPG85-257P/tetR/YB-1 and EPG85-257RDB/tetR/YB-1 cell lines, as observed previously (10).

Global mRNA expression. The microarray used (Affymetrix HGU133 2.0) for global mRNA expression profiling contains 20,000 probe sets corresponding to 14,500 human genes. Gene expression analysis identified several potential target genes of YB-1 in drug-sensitive and drug-resistant gastric carcinoma cells.

Treatment of drug-sensitive EPG85–257P/tetR control cells without anti-YB-1 shRNA sequences with tetracycline resulted in differential regulation of 537 genes distributed on 731 different spots, *i.e.* 537 genes appear to be regulated by tetracycline but independently by YB-1. Tetracycline exposure of drug-sensitive EPG85 257P/tetR/YB-1 cells that express anti-YB-1 shRNAs resulted in differential regulation of 592 genes distributed on 822 spots. Taken together, calculating the relation of YB-1-expressing cells to control cells without YB-1 expression under tetracycline treatment condition to untreated cells, 55 genes appear to be regulated YB-1-dependently.

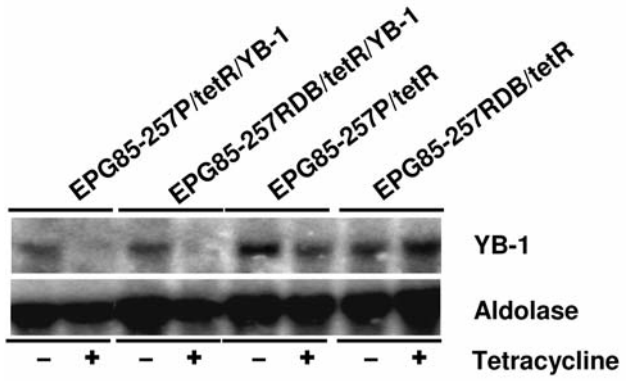

Figure 1. *Northern blot analysis depicting silencing of YB-1 mRNA expression following treatment with tetracycline. As loading control, the Northern blot membranes were probed using an aldolase-specific cDNA. EPG85-257P/tetR, parental EPG85-257P cells expressing the tetracycline repressor; EPG85-257RDB/tetR, multidrug-resistant EPG85-257RDB cells expressing the tetracycline repressor; EPG85-257P/tetR/YB-1, EPG85-257P/tetR cells containing the anti-YB-1 shRNA encoding tetracycline-dependent expression vector; EPG85-257RDB/tetR/YB-1, EPG85-257RDB/tetR cells containing the anti-YB-1 shRNA encoding tetracycline-dependent expression vector.*

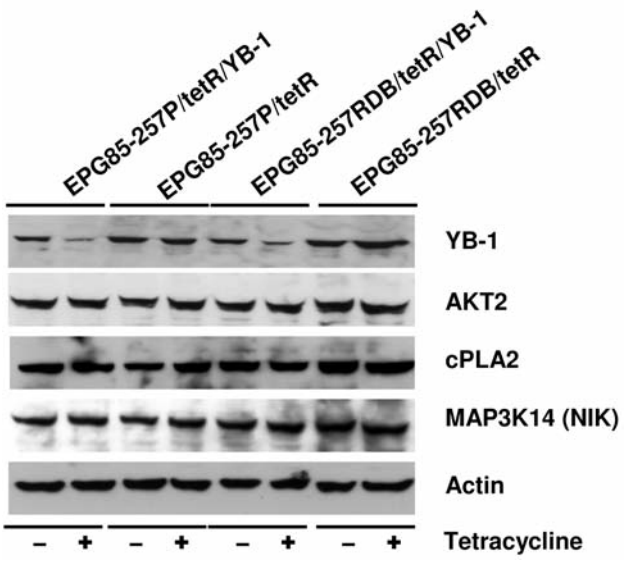

Figure 2. *Western blot analyses of arbitrarily selected proteins (AKT2, cPLA2, MAP3K14) from different pathways following treatment with tetracycline. As control for successful shRNA-mediated YB-1 inhibition, YB-1 expression and as loading control actin expression were analysed. EPG85-257P/tetR, parental EPG85-257P cells expressing the tetracycline repressor; EPG85-257RDB/tetR, multidrug-resistant EPG85-257RDB cells expressing the tetracycline repressor; EPG85-257P/tetR/YB-1, EPG85-257P/tetR cells containing the anti-YB-1 shRNA encoding tetracycline-dependent expression vector; EPG85-257RDB/tetR/YB-1, EPG85-257RDB/tetR cells containing the anti-YB-1 shRNA encoding tetracycline-dependent expression vector.*

In drug-resistant EPG85 257RDB/tetR cancer cells, 636 genes distributed on 950 spots were tetracycline-dependently regulated, and 1000 genes distributed on 1,430 spots were regulated in anti-YB-1 shRNA-expressing EPG85 257RDB/tetR/YB-1 cells. In summary, according to the offset of YB-1-expressing cells without treatment to YB-1 negative cells without treatment against YB-1 expressing cells with treatment to YB-1 non-expressing cells with tetracycline treatment, 364 genes appear to be potentially YB-1-dependently regulated.

Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) list (http:www.genome.jp/kegg/), potentially YB-1-dependently regulated genes were assigned to various pathways, such as the Jak/STAT (janus kinase/signal transducer and activator of transcription), VEGF (vascular endothelial growth factor) and the MAP-kinase (mitogen-activated protein kinase) signaling pathways (Table II) and were randomly used for further investigation. Table II shows a representative selection of potentially regulated genes, related to the identified signaling pathways.

Validation of YB-1-dependent mRNA regulation. Real-time RT-PCR was performed for independent confirmation of the microarray-generated gene expression data. For this approach, some arbitrarily chosen potentially regulated factors were analyzed in detail. Table III shows that the expression levels of the YB-1-encoding mRNA was lower in drug-sensitive and drug-resistant gastric carcinoma cells, but none of the potentially regulated factors was significantly regulated by YB-1.

Validation of YB-1-depending protein regulation. Potential YB-1-dependently regulated factors were also validated at the protein level by applying Western blot analysis. Likewise, three factors (AKT2, v-akt murine thymoma viral oncogene homolog 2; cPLA2, cytosolic phospholipase A2; mitogen-activated protein kinase kinase kinase 14, MAP3K14) from different pathways were selected arbitrarily. As shown in Figure 2, none of the proteins showed differences in expression level to be dependent on YB-1.

Discussion

The multifunctional protein YB-1 was identified as contributing to gene regulation in mammalian cells, including human cancer cells (14-16). YB-1-dependently regulated genes include cancer-associated factors such as the MDR-related drug extrusion pump MDR1/P-gp (8). However, it would appear that not all YB-1-dependently regulated genes in different cancer cells have yet been identified. In particular, in drug-sensitive and drug-resistant cancer cells different genes should be influenced by YB-1. Thus, the potential role of YB-1 in regulation of gene expression in drug-sensitive and drug-resistant cancer cells was investigated by global gene expression analyses in the

Table II. *Genes found to be YB-1-dependently up- or down-regulated in both gastric carcinoma variants.*

Cell variant	Gene symbol	Gene description	Pathways	Fold change†
EPG85-257P/tetR/YB-1	*NFAT5*	Nuclear factor of activated T-cells 5, tonicity-responsive	VEGF, Wnt	14.93
	CHP	Calcium-binding protein P22	VEGF, MAPK, Apoptosis, Wnt	2.83
	ELK1	ELK1, member of ETS oncogene family	MAPK	–8.57
	RASA1	RAS p21 protein activator (GTPase activating protein) 1	MAPK	–17.15
	IL1R1	Interleukin 1 receptor, type I	MAPK, Apoptosis	3.48
	IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	Cytokine signaling, Jak/STAT	–2.14 –2.14
	LEPR	Leptin receptor	Cytokine signaling, Jak/STAT	–17.15
	PIAS2	Protein inhibitor of activated STAT 2	Jak/STAT	–2.14
	SMAD2	SMAD family member 2	Wnt, TGF-β	–3.25
	YB-1	Y-Box binding protein 1	-	–18.4 –13.9
EPG85-257RDB/tetR/YB-1	*SRC*	v-src sarcoma (Schmidt-Ruppin A-2) viral	VEGF	13.93
	AKT2	oncogene homolog (avian)	VEGF, MAPK, Jak/STAT, Apoptosis	–59.71
		v-akt murine thymoma viral oncogene homolog 2		–5.28
	CDC42	Cell division cycle 42 (GTP-binding protein, 25 kDa)	VEGF, MAPK	2.46 2.46
	CHP	Calcium binding protein P22	VEGF, MAPK, Apoptosis, wnt	–2.83
	IFNAR2	Interferon (alpha, beta and omega) receptor 2	Jak/STAT, Toll-like receptor	–2.3
	PIAS2	Protein inhibitor of activated STAT 2	Jak/STAT	–9.19
	IL26	Interleukin 26	Cytokine signaling, Jak/STAT	–6.06
	IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	Cytokine signaling, Jak/STAT	–2.46
	LIFR	Leukemia inhibitory factor receptor alpha	Cytokine signaling, Jak/STAT	–2.3 –4.59
	MAP3K14 (NIK)	Mitogen-activated protein kinase kinase kinase 14 (nuclear factor inducing kinase)	MAPK, Apoptosis	3.25
	MAP2K5	Mitogen-activated protein kinase kinase 5	MAPK, Apoptosis	–6.96
	PLA2G4A (cPLA2)	Phospholipase A2, group IVA (cytosolic, calcium-dependent) (cytosolic phospholipase A2)	MAPK, VEGF	–2.1
	WNT3	Wingless-type MMTV integration site family, member 3	Wnt, Hedgehog	3.73
	YB-1	Y-Box binding protein 1		–27.8 –17.2

†In the case of two different fold change values, the gene-specific sequences were distributed on two or more spots.

presence and absence of YB-1. In these experiments, two well-characterized cell models with tetracycline-dependent RNAi-mediated down-regulation of YB-1 (10) were used. These cell models, EPG85-257P/tetR/YB-1 and EPG85-257RDB/tetR/YB-1, were derived from the human gastric carcinoma cell line EPG85-257P (11) and its drug-resistant, MDR1/P-gp-positive subline EPG85-257RDB (12). Analyses of global mRNA expression profiles in the presence and absence of YB-1 was carried out by microarray technology.

Although microarray analyses identified potential YB-1-regulated genes encoding various proteins of important cellular pathways, these observations were not confirmed by independent methodologies. Neither at the mRNA level nor at the protein level was a significant change in gene expression detected. Since it was reported in many studies that YB-1 has direct gene-regulating features in mammalian

Table III. *Real-time RT-PCR analysis of changes in gene expression.*

Gene	EPG85-257P/tetR/YB-1 (fold change)	EPG85-257RDB/tetR/YB-1 (fold chance)
YB1	–7.4	–8.1
IL6ST	1.63	0.44
PIAS2	1.20	0.39
CHP	1.18	–0.37
SRC	–1.15	–0.31
NFAT5	1.45	1.33
MAP3K14 (NIK)	1.35	1.22
SMAD2	1.75	1.35
AKT2	1.46	1.15

cells (14-16), these findings appear to be astonishing. Reasons for these unexpected data may be the result of weakness of the microarray technology. Discrepancies between microarray measurements and validation experiments, *e.g.* real-time RT-PCR data, have been already described in various settings (17, 18). The fold-change values generated by real-time RT-PCR approximately correspond to one third of the fold-change values measured by the microarray approach. Since the majority of differentially regulated genes was estimated to be about 3-fold regulated by microarray hybridization, a significant difference in gene expression could not be detected. A further aspect may be the physiological variation of the level of gene expression. A physiological variance of 20-30% was suggested to be common in biological samples (19). Consequently, it was reported that it makes no sense to asses an alteration of gene expression level which is less than 4-fold as significant (20). Last but not least, the gene-regulating activity of YB-1 may not be pronounced in the investigated cell model. This absence of gene-regulating activity of YB-1 may be caused by a lack of other cellular factors that are necessary for interaction with YB-1, or by tissue- and cell-specific features of YB-1.

In conclusion, the data of this study suggest that YB-1 is not directly involved in the regulation of mRNA expression in drug-sensitive nor in drug-resistant gastric carcinoma cells.

Acknowledgements

This work was supported by grant no. 01GU0615 of the Bundesministerium für Forschung und Technologie (BMBF). We thank Martina Eickmann for editorial help.

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Received September 7, 2009
Accepted December 10, 2009