The MDR1 gene encoded transmembrane ABC-transporter MDR1/P-glycoprotein can mediate the phenotype of multidrug resistance (MDR), a major obstacle in the clinical management of cancer patients. It was hypothesized that YB-1 is a fundamental regulatory factor of the MDR1 gene in tumor cells and can therewith enhance drug resistance. To analyze the potential impact of YB-1 in MDR cancer cells, two specific anti-YB-1 small interfering RNAs (siRNAs) were designed for transient triggering the gene-silencing RNA interference (RNAi) pathway in the MDR cell lines EPG85-257RDB and EPP85-181RDB as well as in their drug-sensitive counterparts EPG85-257P and EPP85-181P. Since both siRNAs showed biological activity, for stable inhibition of YB-1 corresponding tetracycline-inducible short hairpin RNA (shRNA)-encoding expression vectors were designed. By treatment of the cancer cells with these constructs, the expression of the targeted YB-1 encoding mRNA and protein was completely inhibited following tetracycline exposure. These gene-silencing effects were not accompanied by modulation of the MDR1 expression or by reversal of the drug-resistant phenotype. In conclusion, the data demonstrate the utility of the analyzed RNAs as powerful laboratory tools and indicate that YB-1 is not involved in the regulation of the MDR1 gene or the development of the drug-resistant phenotype in MDR cancer cells.