Comparative Promoter Analysis of Doxorubicin Resistance-associated Genes Suggests E47 as a Key Regulatory Element

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Abstract. *Working under the assumption that up- or down-regulation of genes implicated in chemoresistance may be the result of altered function of regulatory transcription factors (TF), over-represented TF-binding sites of gene lists previously associated with doxorubicin resistance were the target of our search. First, a data warehouse was set up containing 52 genes which were present in at least two gene lists; of those, proximal promoter sequences (1 kb upstream and 0.05 kb downstream of the transcriptional start sites) could be retrieved from genomic databases for 45 genes using the EZ-Retrieve. The TOUCAN tool MotifScanner, which searches the TRANSFAC database, was used to detect TF-binding sites (TFBSs) in our set of sequences. The statistics tool of the Java program TOUCAN was applied to the data with the appropriate expected frequencies file to compare the measured prevalence to a background model. The most significantly over-represented TFBS was that of E47 (p=0.00024, prevalence: 0.2 vs. background: 8.19E-6). In summary, based on the results of our analysis it is hypothesized that the E47 transcription factor may contribute to doxorubicin resistance.*

The anthracycline antibiotic doxorubicin (Adriamycin) is widely used for the treatment of lung, breast, ovarian and gastric cancer, lymphomas and leukemias. Although a number of different mechanisms have been proposed for the cytotoxic effect of anthracyclines, the primary mechanism of its action is likely to be the inhibition of DNA biosynthesis *via* binding to topoisomerase II, consequently conferring S/G2 cell cycle arrest (1-3). The major cause of failure of successful cancer treatment is primary drug resistance or the development of secondary antineoplastic drug resistance. Many different mechanisms of resistance to chemotherapy have been identified, which may act simultaneously, be interconnected and mutually influence each other.

Several studies demonstrated that gene expression profiles of cancer cell lines (4, 5) and primary neoplasms (6, 7) could predict the response to a defined anticancer drug treatment regime. DNA array technology for mRNA expression profiling may provide information about the drug resistance status of a given patient and, thus, offer a chance for individual patient-tailored chemotherapy regimens in the future. For example, van't Veer *et al.* demonstrated that breast cancer prognosis can be deduced from the gene expression profile of primary tumors (8) and Chang *et al.* demonstrated that the gene expression profiles of primary breast cancer could predict the response to docetaxel (9).

The fact that more than 5% of human genes are predicted to encode transcription factors (TF) underscores their importance in cell physiology (10). Transcriptional regulatory regions, the so-called promoter sequences, located before the start codon of each gene, contain short – usually 4 to 10 bases long – TF-binding sites (TFBSs). Once activated, TFs bind to the TFBS and, through interactions with other components of the transcription machinery, promote access to DNA and facilitate the recruitment of the RNA polymerase enzymes to the transcription start site (11). One TF may bind to the promoters of several genes and the promoter region of one gene may contain several TFBSs (12). The involvement of different kinds of TFs in the regulation of a given gene makes possible the integration of

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several signaling pathways; this is called 'combinatorial transcription regulation' (13).

A major new challenge in genomic research is to understand and decipher the mechanisms that regulate the expression of co-regulated gene sets in microarray experiments. An important step in this process is the ability to identify regulatory elements, notably the binding sites in DNA for transcription factors. In our study, we aimed to perform an *in silico* analysis to detect the prevalence of over-represented TFBSs in promoter regions of published gene lists associated with doxorubicin resistance.

Materials and Methods

Source data collection. Our previously published gene lists were re-analyzed and the PubMed (http://www.pubmed.com/) was searched for papers presenting additional gene lists associated with doxorubicin resistance in cell lines using the key words "doxorubicin", "cancer", "gene expression" and "microarray". Those publications where the results were limited to a specific cell line were excluded, as these resistance models could describe cell line-specific resistance mechanisms. To reduce heterogeneity, publications where clinical samples or treatment reponses were investigated were also excluded. All together, three different recent studies (see below) describing gene lists associated with doxorubicin resistance, using different microarray platforms, were analyzed.

In our recent work, the expression profiles of 13 different human tumor cell lines of gastric (EPG85-257), pancreatic (EPP85-181), colon (HT29) and breast (MDA-MB-231) origin and their counterparts resistant to daunorubicin or doxorubicin were contrasted (16). cDNA arrays spotted at Stanford University containing 43,000 cDNA clones (~30,000 unique genes) were interrogated and the top 79 genes best correlated with doxorubicin resistance were identified.

Kang *et al.* performed global gene expression analysis using Affymetrix HG-U133A microarrays and identified differentially-expressed genes associated with the acquisition of chemoresistance to the commonly used drugs 5-fluorouracil, doxorubicin and cisplatin in human gastric cancer of different cell lines (17). The gene expression patterns of ten chemoresistant gastric cancer cell lines were compared with those of four parent cell lines using fold-change and the Wilcoxon's test for data analysis. These authors identified 74 genes differentially expressed in doxorubicin-resistant gastric cancer cell lines

In another study, 30 cancer cell lines were tested for sensitivity to 5-fluorouracil, cisplatin, cyclophosphamide, doxorubicin, etoposide, methotrexate, mitomycin C, mitoxantrone, paclitaxel, topotecan and vinblastine at drug concentrations that can be systemically achieved in patients (18). First, using a resistance index, the cell lines were designated as sensitive or resistant, then the subset of resistant *versus* sensitive cell lines for each drug was compared. Gene expression signatures for all cell lines were obtained by searching Affymetrix U133A arrays. An individual prediction profile for the resistance to each chemotherapy agent was constructed containing 253 genes associated with doxorubicin resistance. The overall accuracy of the predictions in a leave-one-out cross validation was 86%.

Table I. *Over-represented transcription factor (TF)-binding sites before and after Bonferroni-correction.*

TF	n	*p* value	Significant after Bonferroni
E47	9	0.00024	Yes
TAL1alphaE47	6	0.01	No
EGR3	4	0.02	No
NFY	17	0.03	No
HEN1	4	0.03	No
ISRE	9	0.05	No

Sequence extraction. First, proximal promoter sequences (1 kb upstream and 0.05 kb downstream of the transcriptional start sites) were extracted from genomic databases using EZ-Retrieve (19). EZ-Retrieve uses the NCBI UniGene and LocusLink (20), TRANSFAC and TFSEARCH (21) databases. The extracted sequences were saved in FASTA format and then imported into TOUCAN (the sequence file is available on request from the corresponing author).

TFBS identification. The Java program TOUCAN was used for comparative promoter analyses of the selected genes (22). Transcription factors not only bind unique DNA sequences, but also have a degree of non-specificity in their sequence recognition. Thus, whether a TFBS exists in a promoter sequence cannot simply be determined by searching for an exactly matching sequence. Instead, matrices are used that give the different nucleotides various weightings depending on their importance for TF binding. A collection of these matrices exists in the TRANSFAC database (www.gene-regulation.com/pub/databases.html#transfac). The relative occurrences in the list of differentially-regulated genes of a set of TFs (TRANSFAC matrices) have to be compared.

The TOUCAN tool MotifScanner, which searches the TRANSFAC database (23), was used to detect TFBSs in our sets of sequences. The prior (stringency level) was set to 0.1 and the human promoter set of the Eukaryotic Promoter Database (EPD) was chosen as third-order background model. The statistics tool of TOUCAN was applied to the data produced by MotifScanner in combination with the appropriate expected frequencies file (human EPD), thereby detecting over-represented features (showing positive significance values) in the sets of selected genes. Finally, the Bonferroni-correction was subsequently applied to compensate for the effect of multiple testing (Table I).

Results

For the comparative promoter analysis the three different gene lists were combined. Out of the total of 312 individual genes, 52 were repeatedly associated with doxorubicin resistance (see Table II). To increase the accuracy of our model, other genes were dismissed and our attention focused solely on the repeated genes. Then, out of the 52 repeated genes, promoter sequences for 45 genes were retrieved (see Figure 1).

Table II. *Genes associated with doxorubicin resistance in at least two different published gene lists.*

UniGene ID	Affy ID	Public ID	Symbol	Gene title
Hs.102267	204298_s_at	NM_002317	LOX	lysyl oxidase
Hs.125457	221531_at	AF309553	REC14	recombination protein REC14
Hs.126256	205067_at	NM_000576	IL1B	interleukin 1, beta
Hs.130865	214247_s_at	AU148057	DKK3	dickkopf homolog 3
Hs.133892	206116_s_at	NM_000366	TPM1	tropomyosin 1 (alpha)
Hs.153355	212636_at	AL031781	QKI	quaking homolog, KH domain RNA binding
Hs.155223	203439_s_at	BC000658	STC2	stanniocalcin 2
Hs.156346	201292_at	AL561834	TOP2A	topoisomerase (DNA) II alpha 170 kDa
Hs.1594	204962_s_at	NM_001809	CENPA	centromere protein A, 17 kDa
Hs.171466	219518_s_at	NM_025165	ELL3	elongation factor RNA polymerase II-like 3
Hs.1908	201859_at	NM_002727	PRG1	proteoglycan 1, secretory granule
Hs.191583	213599_at	BE045993	OIP5	Opa-interacting protein 5
Hs.199695	212281_s_at	BF038366	MAC30	hypothetical protein MAC30
Hs.23348	210567_s_at	BC001441	SKP2	S-phase kinase-associated protein 2 (p45)
Hs.24529	205393_s_at	NM_001274	CHEK1	CHK1 checkpoint homolog
Hs.250687	205803_s_at	NM_003304	TRPC1	transient receptor potential cation channel, subfamily C, member 1
Hs.257049	204236_at	NM_002017	FLI1	friend leukemia virus integration 1
Hs.259047	203434_s_at	NM_007287	MME	membrane metallo-endopeptidase (CALLA, CD10)
Hs.26471	212745_s_at	AI813772	BBS4	Bardet-Biedl syndrome 4
Hs.279766	218355_at	AF179308	KIF4A	kinesin family member 4A
Hs.279905	219978_s_at	NM_018454	NUSAP1	nucleolar and spindle associated protein 1
Hs.309674	212473_s_at	BE965029	MICAL2	flavoprotein oxidoreductase MICAL2
Hs.311589	205006_s_at	NM_004808	NMT2	N-myristoyltransferase 2
Hs.312102	208951_at	BC002515	ALDH7A1	aldehyde dehydrogenase 7 family, member A1
Hs.315167	219000_s_at	NM_024094	DCC1	defective in sister chromatid cohesion homolog 1
Hs.324470	205882_x_at	NM_016824	ADD3	adducin 3 (gamma)
Hs.334562	203213_at	AL524035	CDC2	cell division cycle 2, G1 to S and G2 to M
Hs.334828	213008_at	BG403615	FLJ10719	hypothetical protein FLJ10719
Hs.344037	218009_s_at	NM_003981	PRC1	protein regulator of cytokinesis 1
Hs.36708	203755_at	NM_001211	BUB1B	BUB1 budding uninhibited by benzimidazoles 1 homolog beta
Hs.388160	209512_at	BC004331	C9orf99	chromosome 9 open reading frame 99
Hs.395771	201432_at	NM_001752	CAT	catalase
Hs.414795	202628_s_at	NM_000602	SERPINE1	serine proteinase inhibitor, clade E, member 1
Hs.418138	212464_s_at	X02761	FN1	fibronectin 1
Hs.42650	204026_s_at	NM_007057	ZWINT	ZW10 interactor
Hs.434953	208808_s_at	BC000903	HMGB2	high-mobility group box 2
Hs.435795	201163_s_at	NM_001553	IGFBP7	insulin-like growth factor binding protein 7
Hs.438231	209277_at	AL574096	TFPI2	tissue factor pathway inhibitor 2
Hs.446406	216870_x_at	AF264787	DLEU2	deleted in lymphocytic leukemia, 2
Hs.489033	209993_at	AA455911	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1
Hs.528669	218662_s_at	NM_022346	HCAP-G	chromosome condensation protein G
Hs.624	202859_x_at	NM_000584	IL8	interleukin 8
Hs.6385	53991_at	AA127623	KIAA1277	KIAA1277
Hs.72550	207165_at	NM_012485	HMMR	hyaluronan-mediated motility receptor
Hs.75277	212479_s_at	AK022815	FLJ13910	hypothetical protein FLJ13910
Hs.77274	205479_s_at	NM_002658	PLAU	plasminogen activator, urokinase
Hs.79078	203362_s_at	NM_002358	MAD2L1	MAD2 mitotic arrest deficient-like 1
Hs.799	203821_at	NM_001945	DTR	diphtheria toxin receptor
Hs.81892	202503_s_at	NM_014736	KIAA0101	KIAA0101
Hs.89497	203276_at	NM_005573	LMNB1	lamin B1
Hs.89714	214974_x_at	AK026546	CXCL5	chemokine (C-X-C motif) ligand 5
Hs.93121	205240_at	NM_013296	GPSM2	G-protein signalling modulator 2

The comparative promoter analysis revealed the E47 transcription-factor as being over-represented in our gene set (p=0.00024, n=9, prior frequency: 8.19E-6). These TFBSs were distributed on eight different genes, of which the SKP2 gene had two E47 TFBSs. The consensus sequence for E47 TFBS is: RCAGNTG (TRANSFAC accession no: R02139).

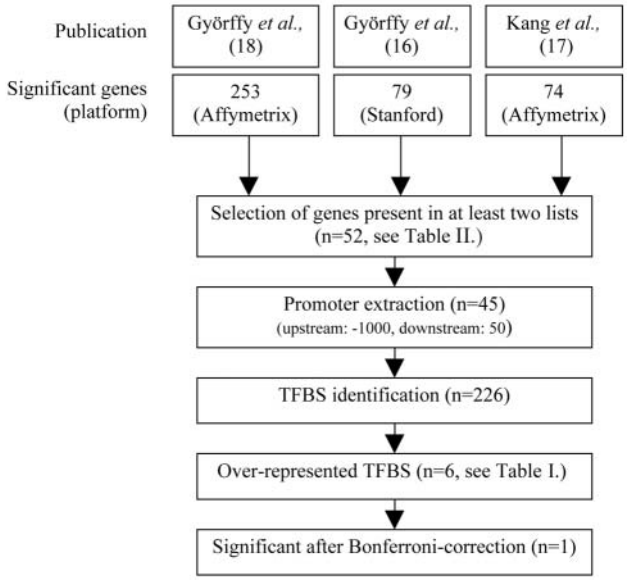

Figure 1. *Overview of the data input and the performed statistical analysis.*

Discussion

In eukaryotes, protein synthesis is regulated at many levels during the transcription from DNA to the mature protein. The first and most important step is the transcriptional initiation, since none of the other steps is possible if no primary RNA transcript is formed. Given a set of genes that share a similar expression profile, a logical step in the analysis is to search for the mechanism that explains their co-expression. The basic assumption is that genes that show a similar expression profile in an experiment might be regulated by the same transcriptional regulators. The activities of many TFs are context-dependent and can be modulated by other modulators nearby. Thus, a single TF can induce the transcription of specific genes while repressing others.

An important step in the identification of relevant TFs in this process is the ability to identify regulatory elements, notably the binding sites in DNA for TFs. First, a co-regulated set of genes is identified, then for each gene in the selected set the promoter or upstream region is identified. Finally, statistically over-represented motifs are retrieved from this set of sequences as potential TFBSs. This approach targets the difficult problem of what to do when little information is available regarding the regulatory system and sequence recognition by the protein.

In our study, the over-representation of the binding site of the E47 TF was identified in the promoter regions of genes associated with doxorubicin resistance. The E47 (accession number: T00207, also called TCF3, BCF-1; E2A.E47; E2A-E47; IEBP1 (rat); and E2A) belongs to the basic helix-loop-helix factors (HLH) class of TFs, to the family of ubiquitous (class A) factors. The E47 contains two trans-activation domains (24), a leucine repeat, a basic region and the helix-loop-helix domain. The basic region contacts the DNA, and the HLH domain is the dimerization interface (25). The dimerization is a prerequisite for DNA-binding. Upon dimerization, the alpha-helical content may significantly increase supporting the four-helix bundle dimerization interface. Upon DNA-binding of the dimer, the basic regions adopt alpha-helical conformation as well. Id helix-loop-helix proteins function as regulators of cell growth and differentiation and, when overexpressed, can induce malignant transformation. Enforced, ectopic expression of the E47 basic HLH (bHLH) protein in human adenocarcinoma cell lines efficiently sequestered endogenous Id proteins as Id-bHLH heterodimers, leading to growth arrest of the cells. E47 plays an active role in tumor cell growth by promoting angiogenesis (26). As the E47 proteins establish a direct transcriptional link between a cell cycle inhibitor, p21(Cip1) and a neutrophic receptor, TrkB, these proteins would play an important role in co-ordinating key events of cell cycle arrest (27). Enforced overexpression of a mutant E47 protein, deficient in transactivation and DNA-binding function, also partially inhibited cell growth. Deregulated expression of Id proteins contributed to the uncontrolled proliferation of tumor cells in colorectal cancer (28). Interestingly, these tumor suppressor properties (29) are more specific for tumor proliferation, not for resistance.

The E47 also interacts with other transcription factors, such as the E12 (Accession no: T00204), Id2 (T01212), Myf-4 (T00520), Myf-5 (T00521), Myf-6 (T00522), MyoD (T00525), Tal-1 (T00790), Tal-1beta (T01448) and Tal-2 (T01630). The E47 is part of the MEF-1 complex; it induces hyper-phosphorylation of MyoD upon association. Additionally, E47 induces transcription of IgH, but also of the IgH-stimulating Oct-2 gene and the recombination-activating genes RAG-1 and -2, thus stimulating IgH rearrangement as well (30). Although a promoter polymorphism in the MHC gene influences the binding of the E47 TF (31), no similar phenomenon has been described in association with drug resistance.

Other transcription factors, *e.g.,* NF-κB and E2F, have already been associated with doxorubicin resistance. The use of agents that block NF-κB function has been highly beneficial in the treatment of tumors in combination with standard anticancer therapies (32). Treatment with BAY 11-7082, an irreversible inhibitor of NF-κB-phosphorylation, induced a higher percentage of apoptosis in vincristine- and doxorubicin-resistant cell lines. The suppression of constitutive NF-κB activity by BAY 11-7082 may be a useful treatment for MDR leukemias (33). The targets of doxorubicin, the topoisomerase II proteins, are direct targets of the E2F TF-mediated transcription and E2F has been proposed to be involved in drug resistance (34).

However, no over-representation for these TFBSs was detected in our study. The question arises of whether a change in the level of transcription of a specific gene is caused by the TF acting directly at the promoter of the gene or through regulation of other transcription factors working at the promoter. It is apparent that these kinds of models are highly complex and difficult to set up. Therefore, the results of this *in silico* analysis will need to be coupled with biological experiments.

Gene regulatory sequences hold the key to understanding how genes are regulated by programmed and environmental signals. Technologies that permit global transcription profiling force the researcher to take a holistic view and consider biological pathways and processes that would otherwise be ignored. The hypothesis that we have proposed should not be considered to be a model that can explain all possible mechanisms of doxorubicin resistance, but must be considered as just one of several influences.

However, our computational approach does not provide information about the functional impact of the identified TFs on gene activation. For the investigation of the TF-DNA complex interaction, experiments should be carried out using functional assays, such as the electrophoretic mobility shift assay (EMSA) or chromatin immunoprecipitation (ChIP). EMSA is based on the observation that protein:DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing polyacrylamide or agarose gel electrophoresis; hence sequence-specific interactions may be investigated. The principle of the ChIP assay is that DNA-bound proteins (including TFs) in living cells can be cross-linked to chromatin by a gentle formaldehyde fixation. Once the proteins are immobilized on the chromatin, the whole protein-DNA complex can be immunoprecipitated using an antibody against the protein in question. The isolated protein/DNA fraction can then be purified for DNA. The identity of the DNA fragments isolated in connection with the protein can then be determined by PCR. Once we have experimental data confirming the binding of the E47 TF, further functional studies can be performed. These include RNA interference and stable transfection to modulate the gene expression and sequencing to identify mutations and SNPs in the E47 gene and in the E47 TFBS.

Conclusion

In summary, based on the results of our computer-simulated analysis of the promoter regions of genes with altered gene expression, we hypothesize that the E47 transcription factor may contribute to doxorubicin resistance in cancer. These results shed a new light on E47, as to date it was only considered to be involved in cell proliferation. Thus, E47 might present a target for effective intervention against doxorubicin resistance.

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