Effects of distinct drugs on gene transcription in an osteosarcoma cell line

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Abstract. Osteosarcoma (OS) is a common cancerous bone tumor which has a detrimental impact on the lives of patients and their families. The present study aimed at investigating the underlying molecular mechanism of various drug treatments pertaining to OS, including dimethyl sulfoxide (DMSO), doxorubicin (DXP), Nutlin-3, actinomycin D (ActD) and etoposide (Eto). Microarray and p53 chromatin immunoprecipitation combined with sequencing (ChIP-seq) datasets of the OS cell line U2OS treated with distinct drugs were acquired from the Gene Expression Omnibus and differentially-expressed genes (DEGs) were screened for alignment analysis. The p53-binding target genes were identified and ChIP-seq and microarray gene expression data were combined to identify directly and indirectly targeted genes. A regulatory network of p53 was constructed with the acquired data. Finally, the Database for Annotation, Visualization and Integrated Discovery was interrogated for annotation of target genes. A total of 212 p53-binding peaks were obtained in the untreated group, whereas thousands of peaks were obtained in the treated groups. In total, ~1,000 target genes were identified in each of DXP, DMSO, Eto and ActD treatment groups, whereas the Nutlin-3 treatment group identified an increased number, with 5,458 target genes obtained. Several common DEGs including *MDM2*, *TP53I3*, *RRM2B*, *FAS* and *SESN1* were targeted by all the drugs with the exception of DMSO. p53 regulated various genes including *EHF*, *HOXA10* and *BHLHE40* in the Nutlin-3 treatment group, whereas p53 regulated *EHF*, *RFX3*, *TRAF40* and *TCF7L2* in the DXR treatment group. The results of the present study indicate that p53 was able to directly regulate target genes including *MDM2*, *TP53I3* and *RRM2B*

or indirectly regulate numerous further genes through several hub genes including *EHF* and *RFX* through various drug treatments in U2OS cells. Furthermore, p53 regulated distinct molecular processes in various drug treatments.

Introduction

Osteosarcoma (OS) is a common cancerous bone tumor most prevalent in children and young adults (1). Specifically, it is a histological form of primary bone cancer derived from primitive transformed cells of mesenchymal origin (2). Numerous patients with OS also suffer from panic attacks and swelling of the lower femur or area directly inferior to the knee, and these symptoms are often exacerbated at night (3). The cause of OS is unknown; however, it is suggested that this disease may be associated with several factors including inheritance, bone dysplasia, germline *p53* mutations and Rothmund-Thomson syndrome (4).

p53 is a tumor suppressor gene that regulates the expression of apoptosis-associated genes when stimulated by specific molecular signals (5). *p53* mutations have been revealed to be associated with the development of OS (6,7). Luo *et al* (8) constructed a regulatory network of OS, and further screened *IL-6* and *BCL2L1* as target genes regulated by p53.

U2OS is a commonly utilized OS cell line. Various chemotherapy drugs, including actinomycin D (ActD), doxorubicin (DXR), Nutlin-3 and etoposide (Eto), have been widely used in OS treatment. Among these drugs, ActD (9), DXR (10) and Eto (11) exhibit direct effects on DNA, inhibiting transcription and promoting apoptosis. However, Nutlin-3 interacts with and disrupts mouse double minute 2 homolog (MDM2), a negative regulator of p53. Inhibiting the interaction between MDM2 and p53 results in an increase in activated p53 and therefore apoptosis (12). In addition, the four drugs can induce cell cycle arrest (13-15). The cell-protective agent dimethyl sulfoxide (DMSO) has also been revealed to affect *p53* (16).

In order to investigate the response of p53 to the various drugs in the U2OS cell line, p53 chromatin immunoprecipitation combined with sequencing (ChIP-seq) and microarray data of ActD, DXR, Nutlin-3 and Eto treatment were downloaded for analysis of molecular mechanism. Differentially-expressed

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Table I. p53 ChIP combined with sequencing datasets.

Author	Gene expression omnibus sample	Description of U2OS cells	(Refs)
Menendez *et al*	GSM1133482	DMSO-treated ChIP	(17)
	GSM1133483	DMSO-treated input	
	GSM1133484	DXR-treated ChIP	
	GSM1133485	DXR-treated input	
	GSM1133486	Nutlin-3-treated ChIP	
	GSM1133487	Nutlin-3-treated input	
	GSM1133488	No treatment ChIP	
	GSM1133489	No treatment input	
Smeenk *et al*	GSM545807	ActD-treated ChIP	(18)
	GSM545808	Etoposide-treated ChIP	

DMSO, dimethyl sulfoxide; DXR, doxorubicin; ActD, actinomycin D; ChIP, chromatin immunoprecipitation.

genes (DEGs) were screened prior to alignment analysis. Finally, the target genes were investigated for the construction of regulatory networks and annotations were processed.

Materials and methods

Data sources. The microarray datasets and p53 ChIP-seq datasets of OS cell line U2OS treated with distinct drugs (17,18) were acquired from the Gene Expression Omnibus (GEO; www.ncbi.nlm.nih.gov/geo) database. The U2OS cell line was treated with various drugs, including DMSO, DXR, ActD, Nutlin-3 and Eto (Table I).

Analytical methods. DEG analysis. The downloaded microarray datasets of ActD and Eto were standardized. Compared with drug treatment groups and the control U2OS cells without any drug treatment, genes with $|\log_2(\text{fold-change})|>1$ were considered to be DEGs. Raw data from DXR, Nutlin-3 and DMSO microarray datasets were processed by Affy analysis of the Bioconductor 2.0 in R (http://www.bioconductor.org/packages/release/bioc/html/affy.html) (19) with P<0.001 and $|\log_2(\text{fold-change})|>1$ considered to indicate DEGs.

Alignment and annotation of gene sequences. Bowtie 2 (version 2.0.0-beta5; http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) (20), a tool for aligning sequencing reads to long reference sequences, was utilized for gene sequence alignment between ChIP-seq and Human Genome hg19. Model-based analysis of ChIP-Seq 2 was applied to identify peaks of transcription factor p53-binding regions (21). The two procedures used the default value as parameter. Peak annotations were processed by CisGenome (version 2.0; http://www.biostat.jhsph.edu/~hji/cisgenome/) (22), an integrated tool for tiling array, ChIP-seq, genome and *cis*-regulatory element analysis. Any genes presenting with a peak located between 2,000 bp upstream and 1,000 bp downstream of the transcription start site was considered to be a p53-binding target gene.

Screening and annotation of target genes. The data gathered from ChIP-seq and expression profile microarray were combined to further screen p53 target genes in the U2OS cell line (promoter range, 2,000±500 bp; the remaining parameters were set using the default values). Subsequently, a p53-centered expression network was constructed. Finally, the Database for Annotation, Visualization and Integrated Discovery (http://david.niaid.nih.gov) (23), an analytical tool for extracting biological information from large lists of genes, was used for annotation of target genes.

Results

Target genes of p53 binding. A total of 212 p53-binding peaks were identified in the untreated group, whereas thousands of peaks were obtained in the treated groups (Table II). Similar numbers of binding sites were identified in the ActD, DXR, Eto and DMSO treatment groups, respectively, with ~1,000 target genes, whereas a total of 5,458 target genes were obtained in the Nutlin-3 treatment group. There were 504 common target genes across the five treatment groups (Fig. 1). Moreover, these target genes were significantly enriched in GO functions associated with positive regulation of apoptosis, positive regulation of programmed cell death and positive regulation of cell death (Table III). Notably, programmed cell death can be divided into several categories including type I (apoptosis) and type II (autophagic death) (24), thus, target genes enriched in positive regulation of apoptosis were different from those enriched in positive regulation of programmed cell death.

Distinct responses to various drugs. A total of five DEGs including *AREG*, *LPP*, *ATF3*, *FAM198B* and *HAPLN1* were revealed across each of the five treatment groups (Fig. 1). Furthermore, a total of 86 common DEGs were obtained from the ActD, DXR, Eto and Nutlin-3 treatment groups, which were classified as Gene Ontology (GO) terms including p53 signaling pathway, cell adhesion and biological adhesion (Table IV). Several common DEGs including *MDM2*, *TP53I3*, *RRM2B*, *FAS* and *SESN1* identified in these four treatment groups were also target genes for p53 binding (Table III).

Table II. Microarray datasets.

Author	Gene expression omnibus sample	Description of U2OS cells	(Refs)
Menendez et al	GSM1131226	No treatment repeat 1	(17)
	GSM1131227	No treatment repeat 2	
	GSM1131228	No treatment repeat 3	
	GSM1131229	DXR-treated repeat 1	
	GSM1131230	DXR-treated repeat 2	
	GSM1131231	DXR-treated repeat 3	
	GSM1131232	DMSO-treated repeat 1	
	GSM1131233	DMSO-treated repeat 2	
	GSM1131234	DMSO-treated repeat 3	
	GSM1131235	Nutlin-3-treated repeat 1	
	GSM1131236	Nutlin-3-treated repeat 2	
	GSM1131237	Nutlin-3-treated repeat 3	
Smeenk et al	GSM552391	Control ActD repeat 1	(18)
	GSM552392	Control ActD repeat 2	
	GSM552393	ActD repeat 1	
	GSM552394	ActD repeat 2	
	GSM552395	Control Eto repeat 1	
	GSM552396	Control Eto repeat 2	
	GSM552397	Eto repeat 1	
	GSM552398	Eto repeat 2	

DXR, doxorubicin; DMSO, dimethyl sulfoxide; ActD, actinomycin D; Eto, etoposide.

p53 indirectly regulates downstream genes through other key genes. p53 was able to activate downstream hub genes following a number of drug treatments (Fig. 2). For example, p53 regulated various genes including *EHF*, *HOXA10* and *BHLHE40* in the Nutlin-3 treatment group, whereas p53 regulated *EHF*, *RFX3*, *TRAF40* and *TCF7L2* in the DXR treatment group. Additionally, p53 was able to indirectly regulate further genes through *TRAF4*, *BHLHE40* and *HOXA10* hub genes (Fig. 2).

Discussion

Owing to systemic chemotherapy, long-term outcomes for patients with OS have improved; however, subsequent progress required further research (2). In the present study, a total of five DEGs were revealed across all five treatment groups including *AREG*, *LPP*, *ATF3*, *FAM198B* and *HAPLN1*. Additionally, a total of 86 common DEGs were obtained in each of the ActD, DXR, Eto and Nutlin-3 treatment groups, certain of which were identified as being associated with the p53 signaling pathway. Following treatment with various drugs, p53 was identified to be able to activate downstream hub genes including *TRAF4*, *BHLHE40* and *HOXA10* which was, in turn, able to affect more genes.

DMSO is a cell-protective agent with limited genetic effects. Of the 86 common DEGs obtained in the four other treatment groups (ActD, DXR, Eto and Nutlin-3), only five were affected by DMSO. In the p53 signaling pathway, DEGs including *MDM2*, *TP53I3* and *RRM2B* were enriched. *MDM2* encodes a nuclear-localized E3 ubiquitin ligase, targets *p53* and further promotes tumor formation (25). Soft tissue sarcoma
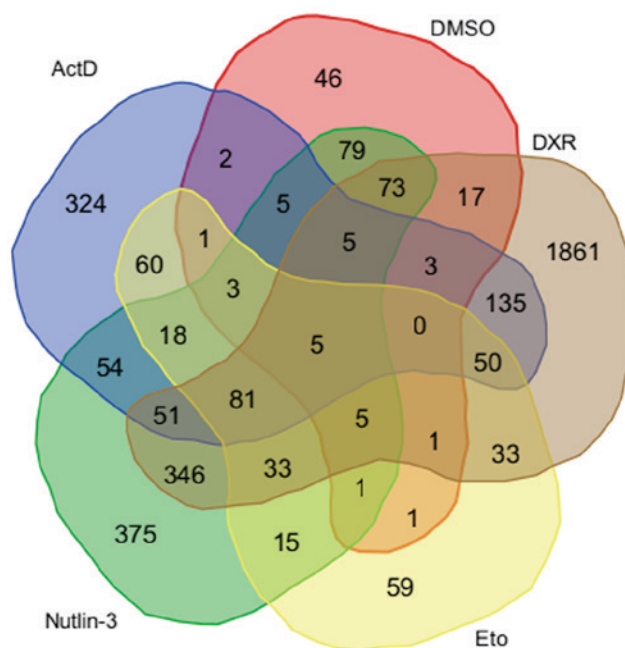

Figure 1. Venn diagram illustrating the number of differentially-expressed gene targets in the DMSO, DXR, Nutlin-3, ActD and Eto treatment groups. ActD, actinomycin D; DMSO, dimethyl sulfoxide; DXR, doxorubicin; Eto, etoposide.

and malignant fibrous histiocytoma are common diseases associated with *MDM2* (26). E3 ubiquitin-protein ligase is able to lead to the degradation of p53 by the proteasome and further

Table III. Gene ontology analysis of p53 target genes.

Gene ontology number	Role	N	Genes	False discovery rate, x10^{-4}
0043065	Positive regulation of apoptosis	32	*ZAK*, *IL19*, *RPS27L*, *RRM2B*, *BCL2L1*, *SRC*, *ZC3H8*, *GPX1*, *AEN*, *FAS*, *PHLDA3*, *FGD3*, *ARHGEF3*, *PTPRF*, *HTT*, *PRKCE*, *VAV2*, *TNFSF8*, *PLEKHF1*, *NOTCH2*, *CDKN1A*, *TNFRSF10B*, *NUPR1*, *BBC3*, *LYST*, *BAX*, *FAF1*, *ABL1*, *DCUN1D3*, *APBB2*, *NGF*, *KALRN*	1.92
0043068	Positive regulation of programmed cell death	32	*ZAK*, *IL19*, *RPS27L*, *RRM2B*, *BCL2L1*, *SRC*, *ZC3H8*, *GPX1*, *AEN*, *FAS*, *PHLDA3*, *FGD3*, *ARHGEF3*, *PTPRF*, *HTT*, *PRKCE*, *VAV2*, *TNFSF8*, *PLEKHF1*, *NOTCH2*, *CDKN1A*, *TNFRSF10B*, *NUPR1*, *BBC3*, *LYST*, *BAX*, *FAF1*, *ABL1*, *DCUN1D3*, *APBB2*, *NGF*, *KALRN*	2.24
0010942	Positive regulation of cell death	32	*ZAK*, *IL19*, *RPS27L*, *RRM2B*, *BCL2L1*, *SRC*, *ZC3H8*, *GPX1*, *AEN*, *FAS*, *PHLDA3*, *FGD3*, *ARHGEF3*, *PTPRF*, *HTT*, *PRKCE*, *VAV2*, *TNFSF8*, *PLEKHF1*, *NOTCH2*, *CDKN1A*, *TNFRSF10B*, *NUPR1*, *BBC3*, *LYST*, *BAX*, *FAF1*, *ABL1*, *DCUN1D3*, *APBB2*, *NGF*, *KALRN*	2.48
0006974	Response to DNA damage stimulus	28	*RAD51C*, *ZAK*, *RPS27L*, *RRM2B*, *SESN1*, *TRIAP1*, *RAD51L1*, *AEN*, *NSMCE2*, *PHLDA3*, *FANCC*, *POLH*, *WRN*, *FOXN3*, *CDKN1A*, *ATXN3*, *RFC3*, *EYA2*, *NUPR1*, *BTG2*, *BBC3*, *BAX*, *DDB2*, *BRE*, *PCNA*, *ABL1*, *GADD45A*, *REV3L*	11.98
0033554	Cellular response to stress	35	*RAD51C*, *ZAK*, *ADORA2B*, *RTN4RL1*, *RPS27L*, *RRM2B*, *SESN1*, *GPX1*, *TRIAP1*, *RAD51L1*, *AEN*, *TPO*, *NSMCE2*, *TRPV4*, *PHLDA3*, *FANCC*, *POLH*, *WRN*, *MAPK10*, *FOXN3*, *RFC3*, *CDKN1A*, *ATXN3*, *EYA2*, *NUPR1*, *BTG2*, *BBC3*, *BAX*, *ATP2A1*, *DDB2*, *BRE*, *PCNA*, *ABL1*, *GADD45A*, *REV3L*	32.40
0006917	Induction of apoptosis	24	*ARHGEF3*, *HTT*, *IL19*, *RPS27L*, *RRM2B*, *VAV2*, *PRKCE*, *TNFSF8*, *PLEKHF1*, *NOTCH2*, *GPX1*, *CDKN1A*, *TNFRSF10B*, *NUPR1*, *BBC3*, *AEN*, *BAX*, *LYST*, *FAS*, *ABL1*, *PHLDA3*, *FGD3*, *NGF*, *KALRN*	93.17
0012502	Induction of programmed cell death	24	*ARHGEF3*, *HTT*, *IL19*, *RPS27L*, *RRM2B*, *VAV2*, *PRKCE*, *TNFSF8*, *PLEKHF1*, *NOTCH2*, *GPX1*, *CDKN1A*, *TNFRSF10B*, *NUPR1*, *BBC3*, *AEN*, *BAX*, *LYST*, *FAS*, *ABL1*, *PHLDA3*, *FGD3*, *NGF*, *KALRN*	98.13

Table IV. Gene ontology and KEGG enrichment analysis of differentially expressed genes in doxorubicin, Nutlin-3, actinomycin D and etoposide treatment groups.

Gene ontology/ KEGG number	Role	N	Genes
hsa04115	p53 signaling pathway	5	*TP53I3*, *MDM2*, *RRM2B*, *FAS*, *SESN1*
0007155	Cell adhesion	11	*HAPLN1*, *PVRL4*, *COL17A1*, *LPP*, *PKP4*, *CYFIP2*, *NINJ1*, *KITLG*, *SLAMF7*, *NEGR1*, *FEZ1*
0022610	Biological adhesion	11	*HAPLN1*, *PVRL4*, *COL17A1*, *LPP*, *PKP4*, *CYFIP2*, *NINJ1*, *KITLG*, *SLAMF7*, *NEGR1*, *FEZ1*
0042981	Regulation of apoptosis	11	*TRIAP1*, *TP53I3*, *NUPR1*, *BTG2*, *BTG1*, *RRM2B*, *FAS*, *SLAMF7*, *NEFL*, *ANXA4*, *TP53INP1*
0043067	Regulation of programmed cell death	11	*TRIAP1*, *TP53I3*, *NUPR1*, *BTG2*, *BTG1*, *RRM2B*, *FAS*, *SLAMF7*, *NEFL*, *ANXA4*, *TP53INP1*
0010941	Regulation of cell death	11	*TRIAP1*, *TP53I3*, *NUPR1*, *BTG2*, *BTG1*, *RRM2B*, *FAS*, *SLAMF7*, *NEFL*, *ANXA4*, *TP53INP1*
0008083	Growth factor activity	5	*TGFA*, *KITLG*, *ESM1*, *AREG*, *GDF15*

hsa, human (*Homo sapiens*); KEGG, Kyoto Encyclopedia of Genes and Genomes.
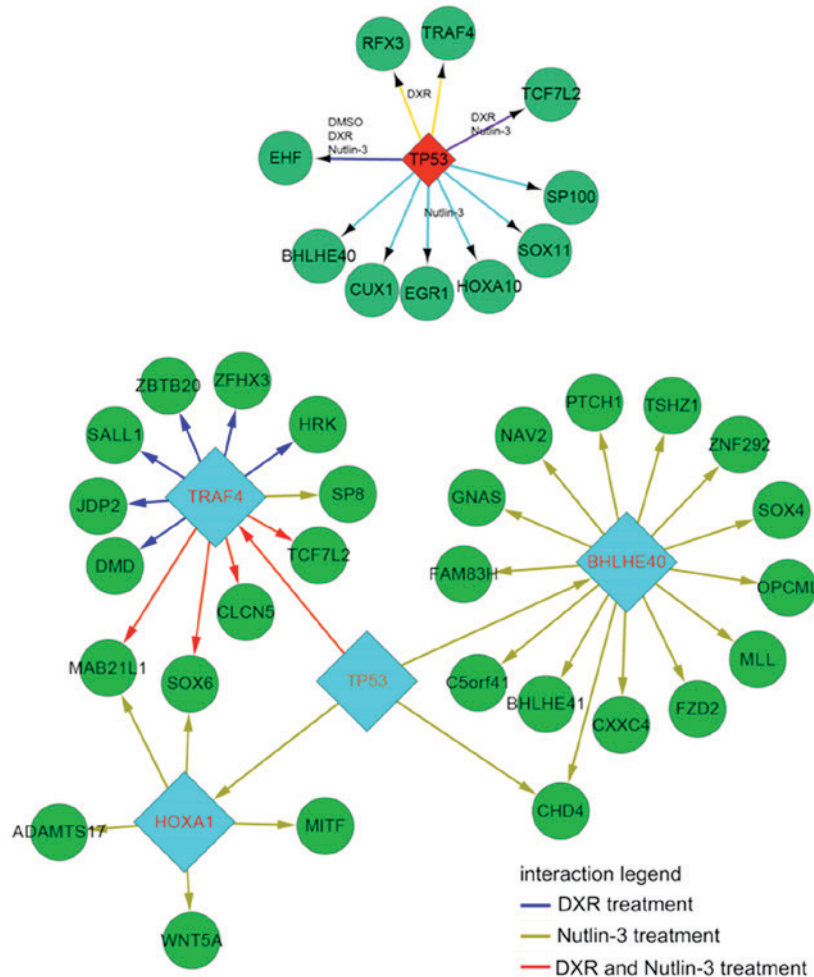

Figure 2. Regulatory network of *TP53*. DXR, doxorubicin; DMSO, dimethyl sulfoxide.

inhibits cell cycle arrest and apoptosis by binding the transcriptional activation domain (27). In addition, *TP53I3* was also differentially expressed in the non-DMSO treatment groups. *TP53I3* is a protein-coding gene which encodes enzymes involved in cellular responses to irradiation and oxidative stress (28). This gene is considered to be induced by p53 and involved in p53-mediated cell death (29). *TP53I3* is transcriptionally activated by p53 through interacting with downstream pentanucleotide microsatellite sequences, and is associated with the number of pentanucleotide repeats. Furthermore, the microsatellite polymorphism is closely associated with the differential susceptibility to cancer (30). Additionally, *RRM2B* encodes the small subunit of p53-incucible ribonucleoside reductase which catalyzes the conversion of ribonucleoside into deoxyribonucleoside diphosphates (31). This gene serves a crucial role in cell survival through repairing DNA in a p53-dependent manner (31). In the process of cell cycle arrest, *RRM2B* also participates in DNA repair by supplying deoxyribonucleotides (32). Therefore, DEGs including *MDM2*, *TP53I3* and *RRM2B* may be target genes for p53 binding.

In addition to the aforementioned genes, certain downstream genes of p53 may also be affected by drugs. The present study revealed that p53 was able to regulate *EHF* which may in turn regulate further genes in the DMSO, DXR and Nutlin-3 treatment groups. *EHF* encodes a protein that is a member of the E26 transformation-specific transcription factor subfamily (33). The encoded protein may participate in carcinogenesis and epithelial differentiation as a transcriptional repressor (34). In addition, a previous study has demonstrated that *EHF* may perform roles in molecular processes including sequence-specific DNA-binding transcription factor activity and sequence-specific DNA binding (35). Additionally, in the DXR treatment group, p53 was able to regulate hub genes including *RFX3* to further regulate more genes. *RFX3*, a member of the regulatory factor X gene family, encodes a transcriptional activator protein (36). This protein is able to bind to DNA with other *RFX* family members (37). As with *EHF*, GO annotations associated with *RFK3* exhibited sequence-specific DNA-binding transcription factor activity (38). Subsequently, p53 was able to indirectly regulate genes through several hub genes including *EHF* and *RFX* in the U2OS cells treated with a number of drugs.

The results of the present study indicates that p53 is able to directly regulate target genes including *MDM2*, *TP53I3* and *RRM2B* or indirectly regulate more genes through several hub genes including *EHF* and *RFX* as demonstrated using various treatments of U2OS cells. Furthermore, p53 may be involved in distinct molecular processes regulated by various drug treatments. However, further experimental analysis is required to confirm these results.

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