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Microarray analysis of Ewing's sarcoma family of tumours reveals characteristic gene expression signatures associated with metastasis and resistance to chemotherapy

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ABSTRACT

In Ewing's sarcoma family of tumours (ESFT), the clinically most adverse prognostic parameters are the presence of tumour metastasis at time of diagnosis and poor response to neoadjuvant chemotherapy.

To identify genes differentially regulated between metastatic and localised tumours, we analysed 27 ESFT specimens using Affymetrix microarrays. Functional annotation of differentially regulated genes revealed 29 over-represented pathways including *PDGF*, *TP53*, *NOTCH*, and *WNT1*-signalling.

Regression of primary tumours ($n = 20$) induced by polychemotherapy was found to be correlated with the expression of genes involved in angiogenesis, apoptosis, ubiquitin proteasome pathway, and PI3 kinase and p53 pathways. These findings could be confirmed by *in vitro* cytotoxicity assays. A set of 46 marker genes correctly classifies these 20 tumours as responding versus non-responding.

We conclude that expression signatures of initial tumour biopsies can help to identify ESFT patients at high risk to develop tumour metastasis or to suffer from a therapy refractory cancer.

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1. Introduction

The Ewing's sarcoma family of tumours (ESFT) represents the second most common primary malignant bone associated tumour in children and young adults. Genetically this tumour entity is characterised by a chromosomal translocation involving the EWS gene (*EWSR1*) on chromosome 22q12 and a member of the ets-transcription factor family, in 85 % of patients the *FLI1* gene on chromosome 11q24, leading to an aberrant EWS-ets transcription factor. Although the outcome of patients with ESFT has been significantly improved due to the implementation of multimodal treatment approaches, the outcome in subgroups of patients still remains poor. Amongst these, patients with primary metastasised tumours have the most unfavourable outcome.[1] In patients with localised disease, poor histological response to neoadjuvant chemotherapy is associated with higher risk to relapse. However, assessment of tumour regression is only possible in the surgical resection specimen after induction chemotherapy and thus will not allow any prediction of outcome at the time of diagnosis.

At the molecular level, secondary genetic changes correlate with patients' outcome. Both overall number of chromosomal aberrations as well as defined structural changes at specific chromosomal regions like chromosome 16q were found to be associated with poor prognosis.[2,3] In addition, loss of p53 function, either by mutation of the gene itself or by other factors like p16/p14ARF impairment, defines a subgroup of patients showing a reduced overall survival.[4–7] Other factors affecting drug sensitivity or molecular factors reflecting the tendency of the tumour to disseminate are not established for ESFT so far.

To systematically analyse the molecular factors governing the two most important clinico-pathological factors for the prognosis of ESFT patients - metastasis and response to chemotherapy - we profiled the global gene expression of 27 primary ESFT samples using Affymetrix HG U133A microarrays and correlated the gene activity to tumour stage and therapy induced tumour regression. Only tumour specimens showing the characteristic EWS gene fusion were included. Because it is not yet possible to predict resistance to chemotherapy in ESFT, an additional aim of this study was to identify a set of marker genes which provides information on the chemosensitivity of the tumour from the initial biopsy. Complementary to the analysis of primary tumour specimens we also established the resistance profile of ten ESFT cell lines to doxorubicin, etoposide, ifosfamide and vincristine (as in the chemotherapy regimen of the international EURO-E.W.I.N.G. 99 trial[8]) respectively, and correlated the corresponding LC50 values to whole genome gene activity as measured by microarray technology.

2. Patients and methods

2.1. Patients

Suitable fresh frozen tumour material from 27 primary ESFT patients was available, which was supplied by the authors' institutions (H.J., E.K., A.E., M.N., G.G., D.T.S.) and nine other German childhood cancer centres. The collective comprised 11 female and 16 male patients, and the median age was 12 years (range 3–35 years). Patients were treated according to the EURO-E.W.I.N.G. 99 protocol including neoadjuvant chemotherapy (vincristine (V), ifosfamide (I), doxorubicin (D) and etoposide (E)). Staging, in order to detect primary metastases, was performed at time of diagnosis according to the EURO-E.W.I.N.G. 99 guidelines. The EURO-E.W.I.N.G 99 trial was organised in compliance with the Declaration of Helsinki, with approval of concerned ethics committees and patient's written informed consent obtained before registration of patients. Grading of histological response to neoadjuvant chemotherapy was performed using the scheme of Salzer-Kuntschik[9] with grade 1 to 3 (<10% vital tumour cells) defined as good responders and grade 4–6 (> 10% vital tumour cells) defined as poor responders. In seven of these patients, the schedule of six blocks of neoadjuvant chemotherapy followed by tumour resection was not applied, so these tumours could only be included in the metastasis-associated expression profile analysis. All tumour specimens could be shown to harbour an EWS/ets gene fusion[10] as detailed in Table 1. Only tissue specimens containing more than 85% tumour cells were employed as confirmed by histopathological examination. *TP53* gene mutation status (exons 5–8) of 17 tumour samples, from which genomic DNA was also available, was determined using standard cycle sequencing techniques. Primers used for bi-directional sequence analysis are described elsewhere.[11]

2.2. Cell lines

All ESFT cell lines used in this study were characterised by EWS gene rearrangement according to Table 2. Cells were grown on gelatin coated culture flasks in RPMI supplemented with 1% Penicillin-Streptomycin (10,000U/ml penicillin and 10 mg/ml streptomycin), 2mM L-glutamine, and 10 % FCS under standard conditions.

2.2.1. Cytotoxicity assay

For these assays, cells were inoculated into 96 well plates in 100 μl medium at cell densities ranging from 5000 to 20,000 cells/well depending on the doubling time. Plates were incubated for 24 h to allow cells to attach. Each experiment included sextuple replicates.

At the time of drug administration, 100 μl of complete medium containing two times the desired test concentrations were added resulting in a final volume of 200 μl and 0.25% DMSO f.c. for drug solubilised in DMSO. Cells were incubated for an additional 48 h and cell viability was measured by standard MTT assay.[12] The cytotoxic effect of drugs was quantified by determining LC50 values, that is the concentration of drug where $100 \times (T_{48h} - T_{0h}) / T_{0h} = -50$ (T = optical density of MTT assay). Median values of replicate analyses and standard deviations were calculated using Microsoft EXCEL®. For these assays, doxorubicin hydrochloride, etoposide, vincristine sulfate (all Sigma, Taufkirchen, Germany), and 4-hydroperoxy-ifosfamide (kindly supplied by Baxter Oncology, Frankfurt, Germany) were employed.

Table 1 – Characterisation of primary tumour specimen

Number	Sex	Age (years)	EWS fusion	Metastatic disease	Response to chemotherapy	p53 Ex5-8	*CDKN2A*
1	f	3	EWS 7 / FLI1 5	0	good	wt	26.0
2	f	7	EWS 7 / ERG 9	0	good	n.a.	14.8
3	f	11	EWS 7 / FLI1 5	0	good	wt	40.3
4	m	12	EWS 7 / FLI1 5	0	good	wt	18.9
5	f	14	EWS 7 / FLI1 6	0	good	wt	11.8
6	m	17	EWS 7 / FLI1 5	0	good	n.a.	16.9
7	f	18	EWS 7 / FLI1 5	0	good	wt	13.3
8	m	19	EWS 7 / FLI1 6	0	good	n.a.	17.6
9	m	26	EWS 10 / FLI 5	0	good	n.a.	19.2
10	m	3	EWS 7 / FLI1 6	0	n.a.	n.a.	9.6
11	m	7	EWS 7 / FLI1 6	0	n.a.	wt	16.9
12	m	11	EWS 7 / FLI1 5	0	n.a.	C141Y	100.0
13	m	12	EWS 7 / FLI1 6	0	n.a.	wt	12.4
14	m	14	EWS 7 / ERG 6	0	n.a.	n.a.	25.2
15	m	17	EWS 7 / FLI1 6	0	n.a.	wt	22.9
16	m	3	EWS 7 / FLI1 6	0	poor	wt	18.7
17	f	6	EWS 7 / FLI1 5	0	poor	wt	30.2
18	f	9	EWS 7 / FLI1 5	0	poor	R248W	45.7
19	f	12	EWS 7 / FLI1 6	0	poor	n.a.	12.7
20	m	35	EWS 7 / FLI1 6	0	poor	n.a.	23.0
21	f	6	EWS 7 / FLI1 6	1	good	wt	10.4
22	m	15	EWS 7 / FLI1 6	1	good	n.a.	10.7
23	f	16	EWS 7 / ERG 6	1	good	n.a.	23.6
24	f	15	EWS 7 / FLI1 6	1	n.a.	wt	20.9
25	m	11	EWS 7 / FLI1 6	1	poor	wt	28.9
26	m	15	EWS 7 / FLI1 6	1	poor	wt	9.1
27	m	17	EWS 7 / FLI1 6	1	poor	wt	25.1

Tumours were derived from 16 male (m) and 11 female (f) patients. All tumour samples were analysed for EWS/FLI1 and EWS/ERG gene fusions. Staging, in order to detect primary metastases, was performed at time of diagnosis according to the EURO-E.W.I.N.G. 99 guidelines (metastatic disease: 1 = metastases, 0 = no metastases). Histological response to neoadjuvant chemotherapy is given as either good (<10% vital tumour cells) or poor (> 10% vital tumour cells). 17 tumour samples were characterised as wild type (wt) for *TP53* exon 5–8 and two show a missense mutation. Expression of the p53 target gene *CDKN2A* is represented by the average of signal values of probe sets 207039_at and 209644_x_at, defining the highest expression to be 100%. n.a. = not available.

Table 2 – Characterisation of ESFT cell lines

	EWS rearrangement	*TP53*-status (45)	Eto [μM]	Dox [μM]	4-H-Ifo [μM]	Vinc [nM]
CADO ES1	t(21;22)	wild type	5	11	6	200
RD-ES	t(11;22)	Arg273Cys	1.5	4.4	20	36
RM 82	t(21;22)	Arg273His	2	2.4	14	3
SK ES1	t(11;22)	Cys176Phe	0.9	2.3	15	3
SKNMc	t(11;22)	truncation	0.7	1.1	7	1
STA ET1	t(11;22)	wild type	0.5	1	11	1
STA ET2.1	t(11;22)	Cys277Tyr	70	1.5	10	1000
TC71	t(11;22)	truncation	1.5	1.3	13	2
VH 64	t(11;22)	wild type	<0.5	0.8	18	8
WE 68	t(11;22)	wild type	1	0.5	11	5
STA ET10	t(2 ;22)	wild type	n.a.	n.a.	n.a.	n.a.

Six of the eleven ESFT cell lines are characterised by a missense or nonsense mutation in the *TP53* gene. LC_{50} values for etoposide (ETO), doxorubicin (Dox), 4-hydroperoxy-ifosfamide (4-H-Ifo), and vincristine (Vinc) were determined after 48 h treatment.

2.3. *Microarray data analysis*

Quality of primary tumour tissue RNA was monitored by capillary electrophoresis (RNA 6000 Nano kit, Agilent, Waldbronn, Germany). The preparation and processing of labelled and fragmented cRNA targets as well as chip hybridisation is detailed elsewhere.[13] Scanning of Affymetrix HG U133A microarrays to achieve chip intensity files was done using Microarray Analysis Suite 5 (MAS5, Affymetrix). ProbeSet annotations used in this study are based on the March 2007 release. Experiment reports were inspected for background, noise, fraction of 'Present Calls', and presence of hybridisation controls. In addition, GeneData Refiner (GeneData, Basel, Switzerland) was used to quality check and import experiments with detection and masking of outliers and array defects, assessment using 3'/5' ratios of housekeeping controls

GAPDH and β-Actin, and condensation of intensities to *Signal* values using 'Affymetrix Statistical (MAS5)' method. In GeneData Analyst (version EPro 1.0.17) normalisation onto an arithmetic mean of 300 was performed (as this minimally shifts the Signal ranges of all experiments). For all bioinformatic calculations on primary tumour tissue samples, only probe sets which were called 'present' by MAS5 for at least one tumour sample were included in our study. This low stringency criterion for valid probe set selection was also used to include the very informative genes, which are switched on or switched off in one of the analysed groups. For comparison of *in vitro* and *in vivo* analysis, only probe sets called present for at least one cell line and at least one tissue specimen were included (12,465 probe sets).

Data files comprising all the processed expression data used in this study can be downloaded from http://home.rz.uni-duesseldorf.de/~k-sch001/download ('Primary-Tumors.xls' and 'Cell-Lines.xls'). Raw data files can be obtained from ArrayEXPRESS database (http://www.ebi.ac.uk/arrayexpress, accession-no E-MEXP-1143).

2.4. Metastasis associated genes and pathways

To identify genes differentially expressed between groups of patients with localised versus metastatic disease, we computed two-sample, two sided *t*-tests between corresponding groups. Genes with a p value less than 0.05 were considered as candidate genes. To avoid loss of substantial numbers of true positive genes, no correction for multiple testing was performed. This list of genes was employed to identify significantly activated pathways by comparing their functional annotations according to the PANTHER classification systems (Protein Analysis Through Evolutionary Relationships, PANTHER Pathways, version 2.0 (May 2007), www.pantherdb.org,[14]) with the whole human genome (Entrez Gene and RefSeq data updated to NCBI's January 10, 2007 release) by the binomial distribution function.[15]

2.5. Genes and signalling pathways associated to chemotherapy resistance

To identify marker genes for the prediction of sensitivity to polychemotherapy, expression data from tumours of 20 patients treated according to the VIDE protocol of the EURO-E.W.I.N.G. 99 trial were available. Cross-validation of classification algorithms (Expressionist, GeneData) was performed to discriminate the groups of responders and non-responders. For this analysis the k-nearest-neighbour algorithm with Positive Correlation as distance function was used, based on a within-set validation, where a randomly chosen part of the input set was not used for training. The misclassification rates are calculated on this test set (averaged over repeated runs).

2.6. Comparison of in vivo and in vitro analysis

Genes related to both the response to polychemotherapy in ESFT patients as well as to mono-drug-therapy for ESFT cell lines were identified by comparing the lists of probe sets covering the 10% of genes showing the highest correlation (or inverse correlation) to *in vivo* or *in vitro* chemo sensitivity, respectively. For primary tumour specimens, these lists were generated by calculating the ratios of median expression values for the group of responders to non-responders. For the *in vitro* analyses, the cell lines were treated separately with each of the four drugs and the obtained log(LC50) values correlated to the gene expression level (log(signal_value)). The first decile of Pearson correlation coefficients (and inverse correlation, respectively) defines the list of genes associated to *in vitro* drug resistance. Lists of genes associated with resistance *in vivo* and *in vitro* were finally screened for significant overlap by Fisher's exact test.

2.7. Induction of p53 target genes by doxorubicin

Cells were treated using 2 μM doxorubicin for 6 h and harvested for RNA isolation. Gene expression was quantified on the LightCycler System (Roche Diagnostics) by qRT-PCR using QuantiTect Primer Assays for *MDM2*, *CDKN1A*, *TNFRS10B*, *BAX*, *LIF*, and *GADD45* together with QuantiTect SYBR Green PCR Kit (both QIAGEN, Hilden, Germany) as described elsewhere.[16] Beta-2-microglobulin (*B2M*) expression was quantified for normalisation of treated versus untreated cells using primers B2Ms 5′-GAATTCACCCCCACTGAAAA-3′and B2Ma 5′-CCTCCATGATGCTGCTTACA-3′.

3. Results

Tumours of all the 27 patients of this study were characterised by a fusion of the *EWSR1* gene to either *FLI1* (24/27, 89%) or *ERG* (3/27, 11%) (Table 1). Genomic DNA to determine mutations of *TP53* exons 5–8 was available from 17 cases. Two cases were identified to carry *TP53* missense mutations including a 9 year-old female with no detectable metastasis at time of diagnosis but poor response tumour to chemotherapy, and an 11 year-old male patient, again with no metastasis, who could not be graded according to Salzer–Kuntschik. Nevertheless, this patient experienced mesenterial metastasis despite polychemotherapy after 5 months. Among known *TP53* target genes, elevated *CDKN2A* ($p16/p14^{ARF}$) expression was observed in these two p53-mutated tumours.

3.1. Metastasis-associated signalling pathways

In order to identify pathways which are of specific interest in the dissemination of ESFT, we defined genes that are differentially expressed in a group of seven metastatic tumours versus 20 localised tumours.

Using *t*-test statistics 1068 out of 15,039 probe sets were assigned to be changed within these two groups of tumours ($p < 0.05$). These 1068 probe sets represented 958 annotated genes according to the NetAffx annotation database (Table 1S-Metastasis-Genes, supplementary data), which correspond to 944 mapped genes in the PANTHER database.

By comparing the functional annotation of these 944 genes with the signalling pathways of all the 25,431 human genes (as represented in the NCBI *H.sapiens* genome database), 29 pathways were found to be over represented ($p < 0.05$) based on the list of differentially regulated genes. Applying correction for multiple testing according to Benjamini–Hochberg,

Table 3 – Metastasis-associated pathways

Pathway	No. of regulated genes	*p*-value
Axon guidance mediated by netrin	7	8.51E-04
PDGF signalling pathway	17	9.12E-04
Huntington disease	15	2.37E-03
Wnt signalling pathway	24	3.48E-03
PI3 kinase pathway	11	6.35E-03
Insulin/IGF pathway-protein kinase B signalling cascade	9	9.43E-03
T cell activation	10	9.55E-03
Apoptosis signalling pathway	11	1.10E-02
Interleukin signalling pathway	14	1.53E-02
Alzheimer disease-presenilin pathway	11	1.96E-02
Ras Pathway	8	2.19E-02
Alpha adrenergic receptor signalling pathway	4	2.38E-02
Cell cycle	4	2.38E-02
Cytoskeletal regulation by Rho GTPase	9	2.46E-02
Angiogenesis	15	2.64E-02
EGF receptor signalling pathway	11	2.65E-02
Inflammation mediated by chemokine and cytokine	19	2.88E-02
Muscarinic acetylcholine receptor 1 and 3 signalling pathway	6	2.96E-02
Axon guidance mediated by semaphorins	5	2.97E-02
Hedgehog signalling pathway	5	2.97E-02
5HT3 type receptor mediated signalling pathway	3	3.02E-02
p53 pathway	10	3.28E-02
Cortocotropin releasing factor receptor signalling pathway	4	3.56E-02
Purine metabolism	2	3.61E-02
5HT4 type receptor mediated signalling pathway	4	3.90E-02
Axon guidance mediated by Slit/Robo	4	4.26E-02
B cell activation	7	4.35E-02

Signalling pathways involved in metastasis of ESFT biopsies according to functional annotation of the PANTHER database (sorted according to *p*-values).

five pathways ($p < 0.0107$) would still be considered to be significantly over represented.

In Table 3 all the 29 pathways are summarised, and these include the pathways involved in PDGF signalling, Wnt signalling, apoptosis signalling, p53, NOTCH signalling, and angiogenesis.

The pathway represented by the highest number of genes involved ($n = 24$, $p = 0.0035$) is the Wnt-signalling pathway indicating the Wnt signalling pathway to be one of the most likely to be critical for ESFT metastasis. A list of the deregulated genes involved in this pathway is supplied in the supplementary data (Table 2S-Wnt-Genes) and includes, among others, cadherin, alpha-catenin and the protein phosphatase 2.

3.2. *Chemo-response*

3.2.1. *Comparison of in vivo and in vitro analysis*

To identify genes related to the response to neoadjuvant chemotherapy in ESFT, resected tumour tissues of 20 patients could be classified as either responder or non-responder according to grade 1–3 and 4–6, respectively, of the Salzer–Kuntschik scheme (Table 1). Genes were ranked according to the ratio of median expression values of these two groups.

In addition, we first investigated which genes *in vitro* are involved in resistance to the drugs of the VIDE protocol of the EURO E.W.I.N.G. 99 trial. For this purpose ten ESFT cell lines were treated with vincristine, ifosfamide, doxorubicin or etoposide in monotherapy and the LC50 values for these drugs were determined after 48 h (Table 2). The expression profiles of these cell lines were determined using the same microarray platform (HG U133A) as we used for the profiling of primary tumour tissue. Then, for each gene and each cytotoxic drug, the correlation coefficient between log(LC50) values and gene expression level (log(signal_value)) was calculated.

Separately for each drug, we compared the top 10% of genes highly expressed in *in vitro* drug resistant tumour cells with the first decile of genes upregulated in primary tumours which did not respond to the VIDE protocol. And vice versa, genes which were both downregulated in resistant cell lines as well as in non-responding primary tumours were also filtered using the 10% cut off criteria. According to this cut off level, fold changes of gene expression level <0.744 or >1.470 were found to qualify these genes to be down-regulated or up-regulated, respectively, in resistant tumours.

For all the four drugs we observed elevated numbers of genes (probe sets) which were related to *in vitro* toxicity as well as being involved in the regression of primary tumours. Although this overrepresentation did not reach significance for the genes down-regulated in refractory tumours, the groups of activated genes all exhibited *p*-values below 0.05 (chi square test) (Table S3a). This was most prominent for genes related to doxorubicin sensitivity. From the 12,465 probe sets involved in this analysis, 163 probe sets corresponding to 154 well annotated genes were found in the first

decile of *in vitro* resistance to doxorubicin and in the top 10% of genes over-represented in chemotherapy refractory primary tumours ($p = 0.00019$, chi square test). Among others, we found members of the carcinoembryonic antigen-related cell adhesion (CEA) family of proteins (*CEACAM1* and *CEACAM6*) and ribonucleotide reductase M2 to be activated, which all are considered to be involved in the effect of anthracyclines. A summary of genes involved in both response of primary tumours to polychemotherapy and *in vitro* toxicity is given in Tables S3b and S3c (Supplementary data).

3.2.2. *In vitro toxicity of VIDE drugs*

Regardless of the comparison to the data of the primary tumour specimens, measuring the $LC50_{(48h)}$ values for the VIDE drugs reveals principle differences between the cell lines depending on the drug supplied.

As a first result of the *in vitro* cytotoxicity study, we observed for vincristine a very broad spectrum of the $LC50_{(48h)}$ values which were spread over three orders of magnitude (1–1000nM). Also for doxorubicin and etoposide the ranges of $LC50_{(48h)}$ values were distributed over more than a ten-fold range (Dox: 0.5–11 μM, Eto: 0.5–70 μM). In contrast to these observations the range of ifosfamide $LC50_{(48h)}$ values was quite homogeneous (4-H-Ifo: 6–20 μM). All LC50 values are summarised in Table 2.

To identify the functional pathways which were associated with drug resistance, again we used the top 10% of genes whose expression was positively or negatively correlated with LC50 values for each drug separately and analysed these genes for pathway annotations according to the PANTHER database. Overall, 77 different pathways were found to be potentially involved in resistance to at least one of the four drugs. Thirty-four of these 76 pathways were found to be relevant to resistance to all the four drugs, including Angiogenesis, Apoptosis signalling pathway, p53 pathway, PI3 kinase pathway, and Ubiquitin proteasome pathway (Table 4 and Table S4, supplementary data).

To further elucidate the relation between *TP53* gene mutation status and p53 target gene expression in ESFT, cell lines were subjected to genotoxic treatment by doxorubicin. Measuring the expression of six known downstream p53 target genes (*CDKN1A, GADD45A, LIF, BAX, MDM2*, and *TNFRS10B*) by qRT-PCR reveals a characteristic pattern of gene regulation depending on *TP53* mutational status in the ESFT cell lines. In

Table 4 – Chemotherapy-resistance associated pathways

Pathway	Dox *p*-value	Ifo *p*-value	Vinc *p*-value	Eto *p*-value
Integrin signalling pathway	1.96E-11	4.39E-06	3.52E-10	2.91E-05
Wnt signalling pathway	8.47E-10	2.82E-05	1.97E-03	3.14E-03
EGF receptor signalling pathway	2.11E-07	2.27E-05	2.14E-05	1.04E-03
Angiogenesis	6.29E-07	2.27E-06	5.48E-07	1.41E-08
PDGF signalling pathway	6.49E-07	3.97E-05	3.76E-04	1.21E-01
T cell activation	1.29E-06	7.03E-05	1.01E-05	3.75E-03
Huntington disease	1.47E-06	2.12E-03	9.71E-04	4.82E-09
B cell activation	2.07E-06	1.71E-05	7.83E-05	1.35E-02
Ras Pathway	5.00E-06	1.08E-06	1.45E-06	5.84E-05
Inflammation mediated by chemokine and cytokine	7.12E-06	8.90E-06	1.89E-05	1.58E-03
FGF signalling pathway	6.38E-05	1.16E-02	2.74E-04	1.11E-04
FAS signalling pathway	1.60E-04	2.20E-02	1.42E-03	5.14E-03
Cytoskeletal regulation by Rho GTPase	2.45E-04	1.30E-02	3.51E-03	9.89E-03
Parkinson disease	3.51E-04	1.24E-05	2.37E-02	8.11E-05
Apoptosis signalling pathway	8.45E-04	2.62E-02	1.20E-04	4.95E-07
Metabotropic glutamate receptor group III pathway	9.27E-04	2.60E-02	3.79E-02	6.71E-02
Metabotropic glutamate receptor group II pathway	1.34E-03	2.48E-02	2.22E-03	2.71E-03
D1/D5 dopamine receptor mediated sigaling pathway	2.01E-03	4.44E-02	3.42E-02	3.68E-02
TGF-beta signalling pathway	2.86E-03	4.59E-03	1.28E-05	1.79E-04
PI3 kinase pathway	3.76E-03	2.31E-04	1.38E-07	2.29E-06
VEGF signalling pathway	4.83E-03	5.84E-04	1.02E-04	2.41E-04
Alzheimer disease-presenilin pathway	5.08E-03	3.88E-04	1.77E-02	4.37E-03
Histamine H1 receptor mediated signalling pathway	7.40E-03	1.89E-02	3.80E-02	1.17E-01
Muscarinic acetylcholine receptor 2 and 4 signalling pathway	7.52E-03	3.98E-02	1.17E-02	4.58E-03
Ubiquitin proteasome pathway	1.15E-02	8.35E-05	3.89E-05	4.13E-02
Endothelin signalling pathway	1.15E-02	1.00E-04	2.48E-03	6.47E-02
p53 pathway	1.20E-02	4.57E-04	2.59E-03	1.06E-03
Cortocotropin releasing factor receptor signalling pathway	1.60E-02	1.45E-02	1.08E-02	1.50E-02
Beta1 adrenergic receptor signalling	1.80E-02	1.61E-02	3.02E-02	6.71E-02
Interleukin signalling pathway	2.00E-02	6.40E-05	3.49E-06	4.03E-04
D2/D3/D4 dopamine receptor mediated signalling pathway	2.10E-02	6.55E-03	1.38E-02	1.17E-02
5HT2 type receptor mediated signalling	2.56E-02	4.89E-02	3.53E-02	1.30E-02
5HT1 type receptor mediated signalling	3.65E-02	3.31E-02	8.93E-03	1.32E-03
Oxidative stress response	4.67E-02	4.18E-02	1.84E-03	9.99E-04

Signalling pathways involved in resistance of ESFT cell lines according to functional annotation of the PANTHER database (sorted according to *p*-values of doxorubicin (Dox) resistance).

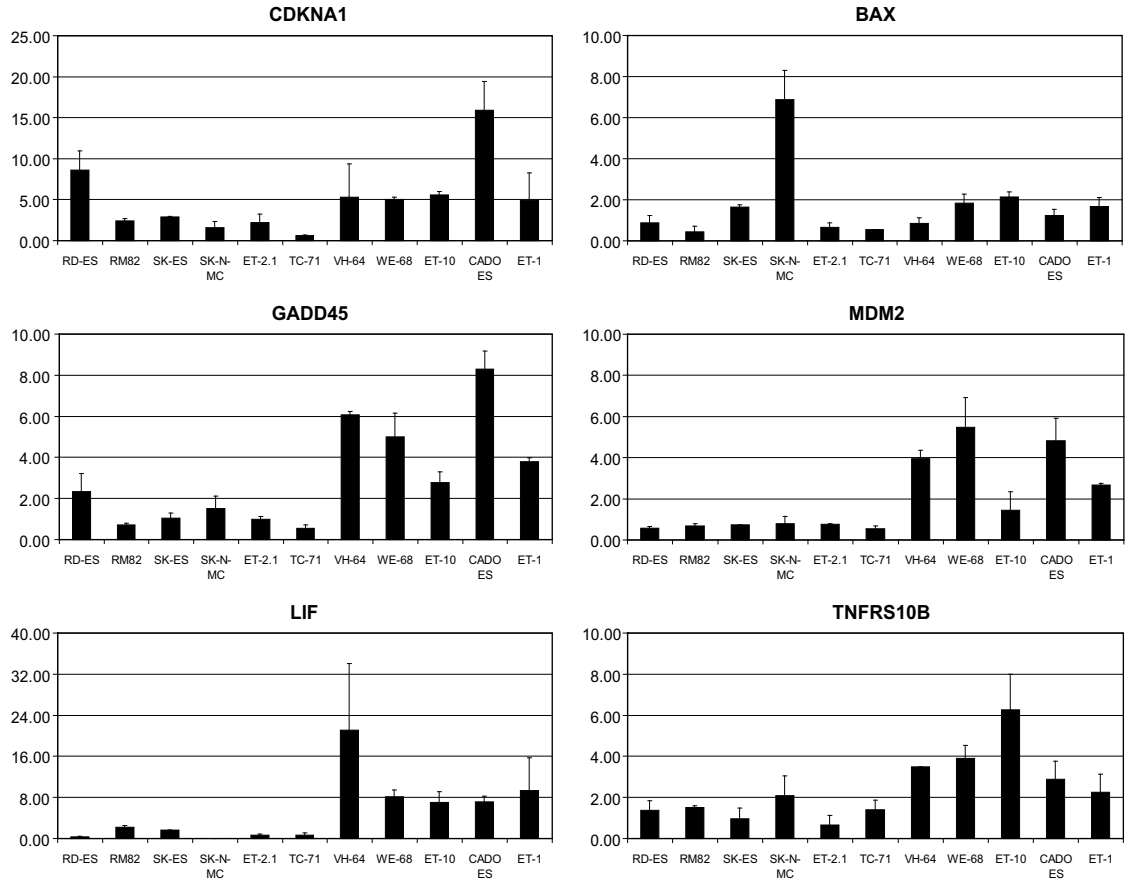

Fig. 1 – Induction of p53 target genes by genotoxic treatment of ESFT cells using doxorubicin. Columns (error bar = SD) indicate the ratio of gene expression as measured by qRT-PCR between unstimulated and doxorubicin stimulated (2 μM, 6 h) cells. Cells harbouring *TP53* mutations (RD-ES, RM82, SK-ES, SK-N-MC, STA-ET 2.1, and TC71) show reduced p53 target gene induction compared to *TP53* wild type cell lines (VH-64, WE-68, STA-ET10, CADO-ES1, and STA-ET1).

this series of experiments an additional *TP53* wild type ESFT cell line (STA ET10) was included.

For all the six genes we observed a reduced induction of target gene expression in the *TP53* mutant cell lines indicating that p53 function as a transcription factor in these cell lines is indeed abolished (Fig. 1). The reduction of transcription activation was significant (*t*-test) for all genes except *CDKN1A* and *BAX*. For *CDKN1A* it has to be noticed that even in the untreated cells the expression of this gene was significantly reduced ($p = 0.02$, *t*-test) in the *TP53*-mutant cell lines.

3.2.3. *Prediction of response to chemotherapy*

Using the tumour expression profile to discriminate between the groups of responders (12 patients) and non-responders (eight patients), an iterated cross validation algorithm led to the identification of a set of 46 probe sets, whose quantification was able to separate between sensitive and non-sensitive tumours without any misclassification in the k-nearest-neighbour analysis.

The genes represented by these 46 probe sets include v-ets erythroblastosis virus E26 oncogene homolog 2 (avian), uracil-DNA glycosylase, ATP-binding cassette, sub-family B (MDR/TAP) member 6, *SPFH2* (SPFH domain family, member 2), vacuolar protein sorting 52 (yeast), and BTB and CNC homology 1, basic leucine zipper transcription factor 2, which are all known to be involved in resistance of cancer cells to chemotherapeutic drugs. A summarisation of all the 46 genes is given in Fig. 2.

4. Discussion

The success of a tumour cell population to develop, survive and progress in an immunocompetent host despite antitumoural drug administration depends on a multitude of factors. All these factors, which finally determine the outcome of a particular patient, can influence the expression pattern of the tumour cells leading to a complex superposition of gene activity. Therefore, to reduce this complexity, the aim of this study was not to differentiate between good and unfavourable outcome within our series of ESFT but rather to describe and to predict the expression pattern of genes directly associated with metastasis and resistance to chemotherapy.

4.1. *Metastasis*

We identified about 900 genes which were differentially regulated between primary metastasised and localised tumours.

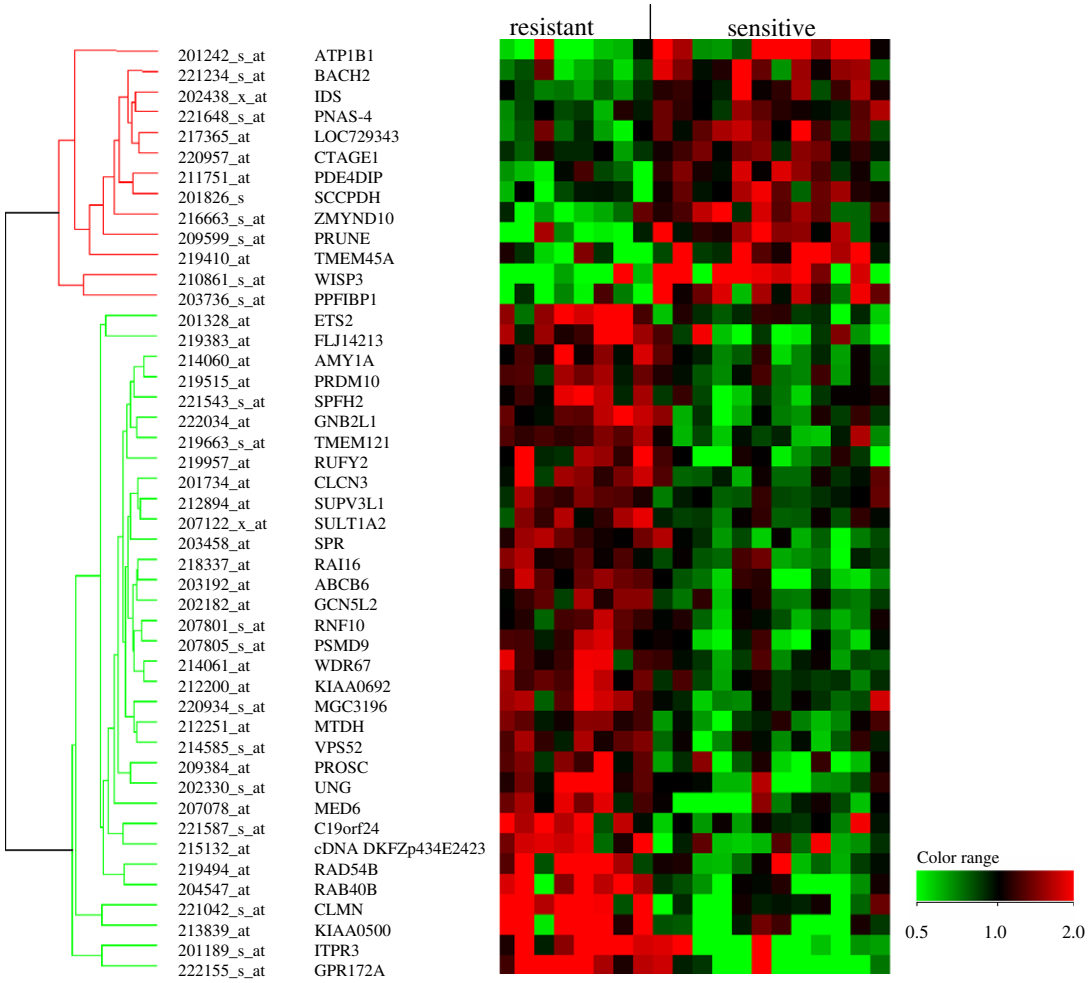

Fig. 2 – Dendrogram and heat map of marker genes predicting response to polychemotherapy in ESFT. Genes (given by Affymetrix ProbeSet-Id and gene symbol) found upregulated are shown in red and downregulated genes are represented in green.

Using functional annotations according to the PANTHER database, pathways of *PDGF* signalling, *WNT1* signalling, apoptosis signalling, *TP53*, *NOTCH* signalling, and angiogenesis were found to be of prime importance for the metastatic phenotype.

While the p53 pathway has been discussed to be involved only in resistance to chemotherapy and radiotherapy in ESFT so far (see below), PDGFR signalling has been shown to regulate tumour cell chemotaxis towards PDGF-BB ligand *in vitro*.[17]

In our comparison between metastatic and localised ESFT, most differentially regulated genes were linked to the Wnt-signalling pathway which plays a key role in cell migration, tissue architecture, and embryonic development.[18] Wnt activation blocks ß-catenin phosphorylation and degradation in the proteasome and enables ß-catenin to accumulate and enter the nucleus where it activates gene transcription. *In vitro*, Wnt-3a induces stabilisation of ß-catenin in ESFT cells which, in turn, leads to a dramatic increase in chemotactic cell migration.[19] Therefore, our data underline the high potential of targeting Wnt-signalling to prevent the dissemination of ESFT cells as it was already shown for other sarcoma samples in preclinical studies.[20]

4.2. *Histological response to chemotherapy*

In several studies the histologic response to chemotherapy as estimated in the surgical specimen was shown to be the most important prognostic factor for ESFT patients.[21,22]

To predict sensitivity to multidrug chemotherapy already in the initial tumour biopsy, we identified a set of 46 marker genes, which allows the discrimination between the groups of responders (12 samples) and non-responders (eight samples) without any misclassification.

Many of these genes are already described for other tumour entities to be involved in tumour cell susceptibility to chemotherapy or tumour progression. ESFT characterised by low sensitivity to polychemotherapy showed, for example, elevated levels of *UNG* (uracil-DNA glycosylase),*ABCB6* (ATP-binding cassette, sub-family B (MDR/TAP) member 6), *SPFH2* (SPFH domain family, member 2), and *VPS52* (vacuolar protein sorting 52 (yeast)). In colorectal cancer *UNG* and *VPS52* were shown to be critical for base excision repair and signal transduction/transport, respectively.[23,24] In breast cancer, elevated mRNA levels of *ABCB6* were recently observed in patients showing residual disease after multimodal chemotherapy

when compared to cases of pathologic complete response.[25] Interestingly, two other overexpressed marker genes, *PROSC* (proline synthetase co-transcribed homolog (bacterial)) and *SPFH2* (SPFH domain family, member 2) were recently described to be co-localised at an amplicon at 8p11.2. The occurrence of this amplicon was both associated with elevated gene expression (especially of *SPFH2*) and correlated with worse prognosis in breast cancer.[26]

In chemo-resistant ESFT we observed a five-fold down regulation of *WISP3* (WNT1 inducible signalling pathway protein 3). WISP3 is a secreted tumour-suppressor protein that inhibits IGF signalling in inflammatory breast cancer, and promotes neoplastic progression in breast epithelial cells if knocked down.[27,28] Since ESFT are known to rely on signalling through the insulin-like growth factor-1 receptor for growth and transformation,[29] we hypothesise that absence of *WISP3* in resistant ESFT supports IGF-I signalling and survival of tumour cells.

Another downregulated gene is the transcription factor *BACH2* (BTB and CNC homology 1, basic leucine zipper transcription factor 2).[30] In BCR/ABL positive chronic myeloid leukaemia cells, low levels of BACH2 were associated with low sensitivity to drugs which represent oxidative stressors like etoposide, doxorubicin, and cytarabine.

In a previous gene expression study on 14 primary ESFT samples, Ohali et al.[31] identified clusters of gene associated with either good or poor clinical outcome. Comparing the 49 genes named in their study with our lists of metastasis- or chemosensibility-associated genes results in an overlap of two genes: hence, downregulation of MYO1C is associated with both poor outcome and metastases at time of diagnosis, while overexpression of zinc finger protein 175 is associated with poor clinical outcome and resistance to chemotherapy.

4.3. *Comparison of in vivo and in vitro analysis*

To further identify genes related to the drug-specific response to chemotherapy in ESFT, we additionally investigated which genes *in vitro* are involved in resistance to one of the drugs of the VIDE protocol of the EURO E.W.I.N.G. 99 trial.

For all the four drugs we found significantly elevated numbers of genes which were related to *in vitro* toxicity as well as being involved in the regression of primary tumours.

Since doxorubicin is included in almost every clinical treatment study on ESFT (reviewed in[32]), resistance to this drug is of high interest. Among the genes showing high correlation to doxorubicin resistance *in vitro* and elevated expression in chemoresistant primary tumour samples we found the ribonucleotide reductase M2 polypeptide (*RRM2*) and *CEACAM6* (CD66c, NCA-90), a member of carcinoembryonic antigen family, So far, immunohistochemical expression analysis of CD66 in ESFT has not been linked to clinical parameters.[33,34] While members of the CEACAM family in general are known to be involved in modulating the immune responses associated with infection, inflammation and cancer (reviewed in[35]), *CEACAM6* was also shown to contribute to the drug resistance phenotype of breast cancer, pancreatic carcinoma, and hepatoma.[36–38] *RRM2* has been reported to be involved in drug resistance in osteosarcoma.[39]

4.4. *In vitro toxicity of VIDE drugs*

For the $LC50_{(48h)}$ values of vincristine, we observed a range of three orders of magnitude indicating that there are subsets of ESFT cells which have to be considered as highly resistant to this drug.. In non-small cell lung cancer, disease progression under treatment using vinca alkaloids was significantly more frequent in tumours showing increased *TUBB3* expression.[40,41] Indeed, the three cell lines characterised by the highest resistance to vincristine (STA ET 2.1, CADO-ES1, RD-ES) also revealed the highest expression of TUBB3 making *TUBB3* an interesting candidate gene for further functional analysis.

Resistance to doxorubicin in our analysis of ten cell lines could be shown to be inversely correlated with the expression of caspase-8. Low levels or even absence of the apoptosis initiator caspase-8 has been reported in subsets of ESFT cell lines ([42]: three out of eight cell lines,[43]: 1/7,[44]: 3/8). For the cell line CADO-ES1, which shows the highest degree of resistance to this drug and no detectable levels of caspase-8 mRNA, methylation of regulatory sequences has been shown to cause down regulation of caspase-8 gene transcription.[42] The clinical impact of caspase-8 on ESFT has, so far, not been investigated; however, caspase-8 expression was at least focally absent by immunohistochemistry in 10 to 15% of clinical tumour samples.[42,45] In our set of 20 primary tumour biopsies, no significant difference between sensitive and resistant tumours could be defined by caspase-8 mRNA levels.

Our data show that *in vitro* as well as *in vivo* the regulation of p53 pathway genes is of high impact on the resistance to genotoxic drugs. While for primary tumour samples expression profiling could be analysed only before induction of chemotherapy, *in vitro* the measurement of p53 target genes after p53-induction by doxorubicin was leading to an even more clear segregation between mutant and wild type samples.

While this manuscript was under review, another study analysing 24 primary ESFT samples by array CGH and gene expression profiling was presented[46] showing that there is a direct relation between poor clinical outcome and genomic instability or rather deregulation of genes involved in chromosome segregation, DNA repair, and cell-cycle control. Regarding the p53 pathway as one of the most significant DNA damage induced gate keepers of the cell-cycle, the results by Ferreira et al. are in conclusive agreement with our findings.

We are aware that, while the impact of the *TP53*-signalling pathway could be underlined by these *in vitro* experiments, the relevance of the other pathways mentioned above also still need functional validation by further knock-out or knock-in assays.

Recently, we were also able to show by immunohistochemistry on 49 additional primary tumour specimens, that patients suffering from tumours that show elevated protein expression of p53 or its antagonist MDM2 (or both) are characterised by a significantly reduced overall survival.[6] Moreover, in other clinical studies, patients suffering from tumours harbouring *TP53* mutations[4,5] were found to be at elevated risk for poor response to chemotherapy and reduced overall survival. Finally, in this study, the two *TP53*-mutated tumours also show the highest *CDKN2A* expression which was recently

shown to be of adverse prognostic impact in ESFT.[7] Therefore, we conclude that including the analysis of p53 signalling of ESFT patients in the routine clinical staging may help to define an important subgroup of high risk patients who may benefit from novel therapeutic strategies.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2008.01.020.

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