Heterogeneous in vitro effects of doxorubicin on gene expression in primary human liposarcoma cultures

Daigeler Adrien *1, Klein-Hitpass Ludger2, Chromik Ansgar Michael3, Müller Oliver4, Hauser Jörg1, Homann Heinz-Herbert1, Steinau Hans-Ulrich1 and Lehnhardt Marcus1

Address: 1Department of Plastic Surgery, Burn Center, Hand surgery, Sarcoma Reference Center, BG-University Hospital Bergmannsheil, Ruhr University Bochum, Bürkle-de-la-Camp-Platz 1, 44789 Bochum, Germany, 2Institute of Cell Biology (Tumor Research), IFZ, University of Essen, Vörhöferstr. 173, 45122 Essen, Germany, 3Department of Surgery, St. Josef Hospital, Ruhr-University, Bochum, Germany and 4Tumor Genetics Group, Max-Planck-Institut für molekulare Physiologie, Otto Hahnstr. 11, 44227 Dortmund, Germany

Email: Daigeler Adrien* - adrien.daigeler@rub.de; Klein-Hitpass Ludger - ludger.klein-hitpass@uk-essen.de; Chromik Ansgar Michael - ansgar.chromik@gmx.de; Müller Oliver - oliver.mueller@mpi-dortmund.mpg.de; Hauser Jörg - joerg.hauser@rub.de; Homann Heinz-Herbert - heinz.homann@rub.de; Steinau Hans-Ulrich - hans-ulrich.steinau@bergmannsheil.de; Lehnhardt Marcus - marcus.lehnhardt@rub.de

* Corresponding author

Abstract

Background: Doxorubicin is considered one of the most potent established chemotherapeutics in the treatment of liposarcoma; however, the response rates usually below 30%, are still disappointing. This study was performed to identify gene expression changes in liposarcoma after doxorubicin treatment.

Methods: Cells of 19 primary human liposarcoma were harvested intraoperatively and brought into cell culture. Cells were incubated with doxorubicin for 24 h, RNA was isolated and differential gene expression was analysed by the microarray technique.

Results: A variety of genes involved in apoptosis were up and down regulated in different samples revealing a heterogeneous expression pattern of the 19 primary tumor cell cultures in response to doxorubicin treatment. However, more than 50% of the samples showed up-regulation of pro-apoptotic genes such as TRAIL Receptor2, CDKN1A, GADD45A, FAS, CD40, PAWR, NFKBIA, IER3, PSEN1, RIPK2, and CD44. The anti-apoptotic genes TNFAIP3, PEA15, Bcl2A1, NGFB, and BIRC3 were also up-regulated. The pro-apoptotic CD14, TIA1, and ITGB2 were down-regulated in more than 50% of the tumor cultures after treatment with doxorubicin, as was the antiapoptotic YWHAH.

Conclusion: Despite a correlation of the number of differentially regulated genes to the tumor grading and to a lesser extent histological subtype, the expression patterns varied strongly; however, especially among high grade tumors the responses of selected apoptosis genes were similar. The presedcribed low clinical response rates of low grade liposarcoma to doxorubicin correspond to our results with only little changes on gene expression level and also divergent findings concerning the up- and down-regulation of single genes in the different sarcoma samples.

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Background
Together with malignant fibrous histiocytoma (not otherwise specified sarcoma, NOS), liposarcoma represents the most common entity of soft tissue sarcomas and accounts for approximately 20% of sarcomas in adults [1-3]. Although surgery and radiation therapy could achieve good results concerning local control, distant metastatic disease remains a therapeutic dilemma limiting survival [4,5]. With a maximum response rate of approximately 20% the effects of cytostatics on liposarcoma are still disappoited [6-8]. The most favoured chemotherapeutics for treatment of advanced soft tissue sarcoma, including liposarcoma, are ifosfamide and doxorubicin, but the data for ifosfamide differ with respect to improvement of local control and survival [9-11]. Although meta-analysis of 14 randomised trials found that doxorubicin treatment was associated with a 10% improvement of recurrence free survival, the overall survival could not be improved [12-14]. In carcinomas, multiple mechanisms of drug resistance on the molecular level have been characterized [15,16] including over-expression of p53 [17-20], MDR1 (multidrug resistance gene 1) [20-22], MRPI (multidrug resistance-associated protein), the induction of DNA repair [20] and many others involving tumor suppressor genes, oncogenes, cell cycle regulators, transcription factors, growth factor receptors, and cell death regulators. Only little is known about the molecular basis of drug resistance in soft tissue sarcomas and studies on the effect of cytostatics on gene expression, especially in liposarcomas [23-27], are rare. Comprehensive knowledge of the differential expression patterns induced by cytotoxic drugs may be useful for examining the molecular basis of drug effects and also drug resistance. Because of the limited comparability of established purchasable sarcoma cell lines to in vivo tumors, we primarily harvested liposarcoma cells from resection specimens, incubated the cultured cells with doxorubicin and evaluated the changes in gene expression with a focus on genes related to apoptotic pathways. To the authors' knowledge, to date there are no studies that examined the effects of doxorubicin on primary human liposarcoma on a molecular basis.

Methods
Primary human liposarcoma tumor samples of at least 1 cm³ were harvested intraoperatively from patients undergoing resection of an already diagnosed liposarcoma and immediately processed under sterile conditions. Seven atypical lipomas (low grade sarcomas), four dedifferentiated, four pleomorphic, three myxoid/round cell, and one myxoid liposarcoma were included. The grading of the tumors ranged from GI to GIII (GI: 4, GII: 8, GIII: 7). The probes were derived from primary tumors in 12, from recurrent tumors in six, and from metastasis in one case. Nineteen primary human liposarcoma cultures were isolated by dissecting the tumor and digesting the minced samples enzymatically with 10 ml each of collagenase and dispase (10 mg/ml). The single cell suspension was depleted of red blood cells and cellular debris by centrifugation through a Ficoll-Hypaque density gradient. Liposarcoma cells were diluted and cultured during the whole experiment with Leibovitz’s L-15 medium, supplemented with 2 mM glutamine and 10% fetal bovine serum in a humidified atmosphere in free air exchange with atmospheric air. Cells were seeded at a density of 2 × 10⁶ in 25 cm² flasks; 24 h later, after having grown to a subconfluent layer, cell cultures were incubated with doxorubicin (0.5 μg/ml) for 24 h and equal volume of PBS as control [28-30].

Oligonucleotide microarray analysis
For microarray analyses we used the Affymetrix Gene Chip platform employing a standard protocol for sample preparation and microarray hybridization that has been described in detail previously [26,31]. Briefly, total RNA was converted into double-stranded cDNA using an oligodeoxynucleotide primer containing the 17 RNA polymerase binding site (5’-GCATTAGCGGCCGAAAT-TAATACGACTCACATATAGGAGA – (dT)₂₁V-3’) (MWG Biotech, Ebersberg, Germany) for first strand synthesis. After generation of double-stranded cDNA from the first-strand cDNA, biotinylated cRNA was synthesized by in vitro transcription using the BioArray High Yield RNA Transcription Labeling Kit (Enzo Diagnostics, New York, USA). Labeled cRNA was purified on RNase columns (Qiagen, Hilden, Germany) and fragmented and hybridized to HG-U133A microarrays (Affymetrix, Santa Clara, USA). The arrays were washed and stained according to the manufacturer's recommendation and finally scanned in a GeneArray scanner 2500 (Agilent, Santa Clara, USA).

Array images were processed to determine signals and detection calls (Present, Absent, Marginal) for each probeset using the Affymetrix Microarray Suite 5.0 software (MAS 5.0; statistical algorithm). The clustering was performed unsupervised. Pairwise comparisons of treated versus control samples were carried out with MAS 5.0, which calculates the significance (change p-value) of each change in gene expression based on a Wilcoxon ranking test. To limit the number of false positives, we restricted further target identification to those probesets, which received at least one present detection call in the treated/control pair. Each single microarray analysis was derived from one cell culture. The treated cells were compared to the control. Probesets exhibiting a signal log2 ratio > 1.0 and a change p-value < 0.004 or a signal log2 ratio < -1.0 and a change p-value > 0.996 (corresponding to 2-fold up- or down-regulation) were identified by filtering using the Affymetrix Data Mining Tool 3.0 (Table 1). Additionally unsupervised clustering was performed between the 19 control tumor samples.
Table 1: Summarized patients' data

| Patient | Gender | Age at operation | Site | Size in cm | Histological subtype | Responder type | Grading | Specimen character | Previous radiation | Previous chemotherapy |
|---------|--------|------------------|------|------------|----------------------|----------------|---------|-------------------|--------------------|-----------------------|
| 1       | female | 69 years         | lower arm | 4.5 × 3.5 × 2.2 | atypical lipoma with partly dedifferentiated areas | low | G2 | local recurrence | no | no |
| 2       | female | 74 years         | Thigh | 14.5 × 7.5 × 9 | myxoid/roundcell liposarcoma | high | G3 | primary tumor | no | no |
| 3       | female | 70 years         | upper arm | 3 × 5 × 6 | atypical lipoma | low | G1 | primary tumor | no | no |
| 4       | male   | 74 years         | Thigh | 16.5 × 9 × 7 | dedifferentiated liposarcoma | medium | G2 | primary tumor | no | no |
| 5       | male   | 38 years         | Knee pelvis retro-peritoneal | 8.3 × 4 × 7 | myxoid liposarcoma | high | G3 | primary tumor | yes | no |
| 6       | female | 58 years         | Thigh | 7 × 14 × 9 | myxoid/roundcell liposarcoma | medium | G2 | primary tumor | no | yes |
| 7       | male   | 37 years         | Thigh | 11 × 8 × 4 | pleomorphic liposarcoma | high | G3 | primary tumor | no | no |
| 8       | female | 85 years         | lower arm | 10 × 3 × 5 | atypical liposarcoma | low | G2 | local recurrence | yes | no |
| 9       | male   | 76 years         | Thigh | 3.5 × 3 × 3 | dedifferentiated liposarcoma | high | G3 | local recurrence | yes | no |
| 10      | female | 57 years         | Thorax | 4.9 × 4 × 3 | pleomorphic liposarcoma | high | G3 | local recurrence | no | no |
| 11      | female | 76 years         | Thigh | 38.5 × 17.5 × 6 | atypical lipoma | low | G1 | primary tumor | no | no |
| 12      | female | 74 years         | Thorax | 7 × 6 × 4 | dedifferentiated liposarcoma | medium | G2 | primary tumor | no | no |
| 13      | female | 70 years         | Thigh | 1.9 × 1.3 × 1 | pleomorphic liposarcoma | medium | G2 | residual tumor | no | no |
| 14      | male   | 70 years         | Thigh | 9 × 3 × 6 | atypical lipoma | medium | G1 | primary tumor | no | no |
| 15      | male   | 60 years         | Thigh | 7.5 × 6 × 5.5 | pleomorphic liposarcoma | high | G3 | primary tumor | no | no |
| 16      | female | 78 years         | Thigh | 13 × 10 × 6 | dedifferentiated liposarcoma with partly dedifferentiated areas | high | G2 | local recurrence | yes | no |
| 17      | female | 67 years         | Thigh | 35 × 15 × 12 | atypical lipoma | low | G1 | primary tumor | no | no |
| 18      | male   | 60 years         | upper arm | 9,9 × 7 × 7 | dedifferentiated liposarcoma | medium | G2 | local recurrence | yes | no |
Genes associated with apoptotic pathways were selected based on Gene Ontology (GO)-analysis [32]. Expression changes were correlated to the grading and the histological sub-entity of the tumors. Only tumor samples were included in the final analysis whose gross sections were diagnosed as liposarcomas by an experienced soft tissue pathologist.

According to the number of differentially expressed genes after incubation with doxorubicin, liposarcomas were categorized into high (n > 2000), intermediate (100 < n < 1000) and low responders (n < 100). The results were uploaded to NCBI GEO, number GSE12972 [http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12972](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12972).

**Real-time PCR for microarray data validation**

Microarray data validation was performed for selected gene products with relevant up-regulation in more than ten out of the 19 liposarcoma probes (CD40, CDKN1A, FAS, GADD45A, NFKBIA, PAWR, TNFAIP3, and TNFRSF10B) or relevant down-regulation in at least ten probes (YWHaH, PPP3CA, and ITGB2). CD14 and TIA were not tested because no high quality PCR assays were purchasable.

Total RNA (2 μg) was reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR was done with a 7900HT SDS system (Applied Biosystems) in 20 μl reaction volume containing 1× Master Mix, 1 μl assay and cDNA equivalent to 2 ng total RNA. All reagents and realtime PCR assays (m1 type only; CD40_Hs00386848_1, CDKN1A_Hs00355782_m1, FAS_Hs00236330_m1, GADD45A_Hs00169255_m1, ITGB2_Hs00164957_m1, NFKBIA_Hs00153283_m1, PAWR_Hs00169332_m1, PPP3CA_Hs00174223_m1, TNFAIP3_Hs00234713_m1, TNFRSF10B_Hs00366272_m1, YWHaH_Hs00607046_m1) used were purchased from Applied Biosystems. Reactions were performed in duplicates and analysed by the deltadeltaCT method. Human GAPD was used for normalization. Pearson correlation of the microarray and the rtPCR data was calculated using SPSS Version 15.0 for Windows (SPSS Inc., Chicago, USA).

**Results**

Sixteen tumors were located at the extremities, three at the trunk. The tumors diameters ranged from 1 cm to 38.5 cm. Twelve primary cultures were harvested from primary tumors, 6 from local recurrences, and 1 from a metastatic tumor. The majority of tumors were high grade (G2-3); only 4 were diagnosed low grade (G1). Atypical lipoma or highly differentiated liposarcoma was the most common histological subentity (n = 7) followed by dedifferentiated (n = 4) and pleomorphic (n = 4), myxoid/roundcell (n = 3) and myxoid (n = 1) liposarcoma. All low grade tumors were diagnosed as atypical lipoma, whereas 3 atypical lipomas were categorized as G2 tumors because of aggressive growth or localized areas of dedifferentiation. The other subentities were all categorized as high grade tumors (G2-3). A summary of the patients’ data is given in table 1.

Hierarchical clustering of expression profiles from untreated samples did not clearly separate according to histological subtype or grading, but revealed two major branches, which showed some enrichment for G3 (left) and G2 tumors (right) (fig. 1).

According to the number of differentially expressed genes n after incubation with doxorubicin, the 19 liposarcomas were categorized into high (n > 2000), intermediate (100 < n < 1000) and low responders (n < 100). All poorly differentiated (G3) tumors were high responders; G2 tumors were predominantly intermediate responders; and, most of the G1 tumors were low responders. A heatmap of all differentially expressed genes after 24 h of doxorubicin treatment shows heterogeneous response patterns (fig. 2).

Correlating the overall expression changes with the histological subtype showed that most of the atypical lipomas were low responders (5 low, 1 medium, 1 high), dedifferentiated were predominantly medium responders (3 medium, 1 high), most myxoid/roundcell and myxoid liposarcomas as well as the pleomorphic liposarcomas were high responders (3 high, 1 medium each). The high grade sarcomas (G3) clustered closely together.

The alteration of gene expression related to apoptotic pathways correlated to the categorisation given above. Low responders also did also not respond with relevant gene expression changes of "apoptosis genes" whereas the high responders showed a significantly different gene expression profile concerning apoptosis related genes compared to the untreated control. In all, we found 464 genes with expression changes that are related to apoptotic pathways. The single genes that were differentially expressed in the medium and high responder group only partly overlapped with the low responder group. The heterogeneity of the response patterns of apoptosis related genes is illustrated in figure 3.

Although the diversity of changes in gene expression was large, some apoptosis related genes showed similar expression changes in the tumor samples, especially the high grade tumors (G2 and G3) or high responders. Figure 4 focus on expression changes in these genes. The apoptosis related genes most often affected by doxorubicin treatment are mentioned below. Due to their large number, we only refer to the genes that were differentially (up- or
Figure 1 (see legend on next page)
Figure 1 (see previous page)
Unsupervised hierarchical cluster analysis of the 19 primary tumor cell cultures without doxorubicin treatment.
Gene signal intensities were normalized to the mean signal of all samples, log2 transformed and subjected to hierarchical clustering (UPGMA, Spotfire) and correlation as a similarity measure. Horizontal rows represent individual genes; vertical columns represent individual samples. Black indicates average signal intensity, brightest red ≥ 4-fold up-regulation, brightest green ≥ 4-fold down-regulated gene expression relative to the mean. Only probesets receiving P detection calls in 6 or more samples and a stander deviation of normalized signals > 0.2 were considered. (7239 retained). The dendogram at the top of the matrix indicates the degree of similarity between tumor samples (the higher the dendogramm, the lower the similarity). Two major clusters were identified as indicated. MR: Myxoid/Roundcell Liposarcoma, PL: Pleomorphic Liposarcoma, AL: Atypical Lipoma, ML: Myxoid Liposarcoma, DL: Dedifferentiated Liposarcoma, low: low responder group, medium: medium responder group, high: high responder group.

Discussion
Gene expression profiling has already been helpful in categorizing distinct subtypes of sarcomas by profile clustering [33-35] and identifying subtype specific changes in gene expression in liposarcoma, e.g. abnormal expression of cell cycle regulators in FUS-DDIT3 carrying liposarcomas [34,36-43] and even provided potential targets for new therapeutic agents like important mediators in cell cycle regulation, e.g. MDM2 [44-48].

Gene expression profiling studies on liposarcomas have already shown that this entity presents a somewhat similar expression pattern with malignant fibrous histiocytoma and leiomyosarcoma [31,49] and that highly differentiated lesions cluster with lipoma whereas the dedifferentiated tumors cluster with myxoid/round cell liposarcomas [50]; however no clear correlation between expression patterns and histological subtype could be detected [37]. Another difficulty in the assessment of gene expression profiles is the inter- and intra-tumoral heterogeneity. Several subtypes with different expression patterns and histologic features can often be found within one same tumor [51,52].

Liposarcomas are classified into several types based on histological findings and cytological aberrations – well differentiated (atypical lipoma), dedifferentiated, myxoid, round cell tumors and pleomorphic. The risk of distant metastasis grows with the grading of the lesion to up to 75% in pleomorphic sarcoma. Myxoid tumors with a greater than 5% round cell component, most dedifferentiated, and pleomorphic liposarcomas are considered high grade lesions [4,53-55].

There are no markers to clearly identify liposarcoma cells. S100, CD34, and others may be helpful (as well as cytogenetic techniques) as they can identify aberrations indicating myxoid/round cell sarcoma; however, they cannot identify liposarcoma cells with absolute certainty [56]. In our series, we relied on a proper tumor dissection and preparation of the specimens to ensure, the isolated tissue mainly consisted of liposarcoma cells as previously described by Sreekantaiah et al. [57] and Lehnhardt et al [58], but have to admit that results may be partly falsified by residual tumor stroma cells accidentally co-cultivated within the liposarcoma samples.

Liposarcoma cells showed diverse gene expression patterns before and after incubation with doxorubicin. Tumors of the same histologic subgroup did not cluster together concerning their overall gene expression. The correlation of the number of differentially regulated genes to the tumor grading, and, to a lesser extent, to the histological subgroup after doxorubicin incubation, may be caused by the tumor associated up-regulation of cell metabolism and the therefore greater effect of any interference. The overall expression patterns and the ones of the apoptosis related genes were also very heterogenous (fig. 2, 3). This finding is concordant to the results of other studies with soft tissue sarcoma cells [59] and may partly be explained by the known inter- and intratumoral heterogeneity in soft tissue sarcomas [51,60].

Interestingly the myxoid and the myxoid/round cell liposarcomas clustered together in figure 2 and 3 except from the tumor 7 that was pre-treated with chemotherapy.
Figure 2 (see legend on next page)
If that is a coincidence or may be interpreted as a kind of selectional process that could have eliminated the high grade parts of the tumor leaving the residual to cluster closer to the low grade sarcomas can not be determined.

The predescribed low clinical response rates of low grade sarcoma correlate to our findings that low grade liposarcoma, especially atypical lipoma, showed almost no response to doxorubicin on gene expression level [61]. However, some expression changes in response to doxorubicin treatment, observed especially in the high responder and in the high grade group were similar and are focussed on the figure 4. According to the large number of apoptosis related genes, we identified the first five ones that were differentially expressed in more than 50% of the samples and limit further explanation to these with special reference to apoptotic function in sarcoma or previous reports concerning doxorubicin treatment, although the understanding of their exact functions is limited. The up-regulated genes beyond the mentioned five are summarized in table 5.

**Up-regulated genes**

As previously shown, doxorubicin could overcome TRAIL resistance in a variety of sarcoma cell lines. An up-regulation of the TRAIL-R2, increasing the susceptibility to apoptosis inducing agents such as TRAIL, may be a possible explanation [62-65], as we found the Trail receptor 2 gene expression increased in 15 probes.

The second most frequently up-regulated gene was cyclin-dependent kinase inhibiton 1A (*CDKN1A*), a downstream target of p53, which has already been shown to be involved in cell cycle arrest and apoptosis induction by doxorubicin in sarcoma cells. *CDKN1A* can act as a positive regulator of senescence-like terminal proliferation arrest, but its function seems neither sufficient nor absolutely required for a treatment response to doxorubicin in tumor cells, especially soft tissue sarcoma [66-70].

*GADD45A*, a potent inhibitor of the c-Jun N-terminal kinase (JNK) cascade and *NFKBIA*, inhibits transcription factors associated with tumor growth [71-74] and was up-regulated by doxorubicin in 12 probes. In a variety of soft tissue sarcoma cell lines *GADD45A* was found to increase cell cycle arrest and apoptosis [Zhu, 2008 #165]. For rhabdomyosarcoma, increased *GADD45A* has previously been associated with less aggressive tumor behaviour [75].

**FAS**, a member of the tumor necrosis factor receptor family, seems to be an important mediator in doxorubicin induced apoptosis. Its effects were shown to be dependant on metalloproteinases in soft tissue sarcoma and Ewing sarcoma. These metalloproteinases have been associated with aggressive tumor behaviour and may promote invasiveness and the occurrence metastasis of malignant cells [76-78].

Another member of the TNF receptor superfamily, *CD40*, was also up-regulated by doxubicin in our experiments. To the authors’ knowledge, *CD40* has not yet been associated with doxorubicin treatment in liposarcoma, but its expression in soft tissue sarcoma was associated with an unfavourable outcome [79], whereas Lodge et al. could show a beneficial effect of antibody mediated CD40 activation in elimination of fibrosarcoma in nude mice [80].

**Down-regulated genes**

Among the down-regulated genes related to apoptotic pathways *CD14*, a receptor marking apoptotic cells was found in 8 probes. Increased *CD14* has been associated with apoptosis induction and cell clearance, especially mediated by macrophages, but is expressed by a variety of other cells too [81-83]. Its role in terms of cell death mediation in sarcoma cells has not yet been examined, therefore interpretation of this finding remains difficult.

*TIA-1*, which encodes an RNA-binding protein with translation-regulatory functions has already been reported to be up-regulated in tumor specimens post-treatment with TNF alpha in soft tissue sarcomas. It was further hypothesized that *TIA-1* could mediate death receptor mediated apoptosis in soft tissue sarcoma and that its overexpression
Figure 3

Heatmap of expression changes of genes related to apoptotic pathways after incubation with doxorubicin for 24 h. Horizontal rows represent individual genes; vertical columns represent individual samples (left to right: low responders to high responders). Color range: Brightest red (Change call increased (change p-value < 0.002) and Signal Log Ratio > 1): SLR = >2 (4x or higher), Black: SLR = 0 (no change); not visible as a consequence of the filtering process, Brightest green (Change call decreased (change p-value > 0.998) and Signal Log Ratio < -1): SLR < -2 (0.25x or smaller). Grey: no value (requirements for a reliably measured target not met). Calculated in Affymetrix comparison analysis (MAS5.0 algorithm) and at least one present call in the two single array analyses compared in the comparison analysis.
sion might sensitize endothelial cells to proapoptotic stimuli present in the tumor microenvironment and enhance NK cell cytotoxic activity against cancer cells [84].

YWHAH, or 14-3-3 eta, is a member of the dimeric 14-3-3 family of signal transduction proteins that specifically binds to phosphorylated serine on a variety of signalling molecules, such as Bcl-2, MDMX, and Bax, thereby promoting cell survival and acting antiapoptotic in several tumor cells [85-87]. On the other hand, it is supposed to be associated with tumorigenesis through its binding interaction with gremlin1 [88]. Therefore the issue of further studies should be awaited before interpreting this finding.

PPP3CA (CCN1/Cyr61) is susceptible to various growth factors and promotes cell proliferation, adhesion, and differentiation and plays important roles in angiogenesis. Additionally, PPP3CA has been associated with tumorigenesis. It was reported that PPP3CA exerts its functions via interacting with integrins as well as heparan sulfate proteoglycan. By activating NF-kappaB and tyrosine kinase signalling pathways, PPP3CA is not only able to control cell growth, but also induce or suppress apoptosis in a cell type-specific manner [89,90]. To the authors' knowledge, it has so far not been reported in context with liposarcoma or doxorubicin treatment.

Integrin B2 (ITGB2) is known to play a role in mediating apoptosis [91] and chemotherapy resistance. Although it is widely attributed to white blood cells, it is also expressed in a variety of other benign and malignant cells and seems to play a major role in cell invasion and migration [92].

**Conclusion**

In summary, pro- and antiapoptotic genes were found up- as well as down-regulated with a dominance of up-regulation of proapoptotic genes. The heterogeneous expression profiles reflect the heterogeneous reaction of liposarcomas to doxorubicin therapy. A lot of genes we found differentially expressed have not yet been associated with apoptosis in liposarcoma or doxorubicin treatment. Therefore it is not possible to relate our findings to other studies. Our study shows that the low clinical response rates of highly differentiated liposarcoma correlate to minimal changes in the expression patterns and that only high grade tumors, especially myxoid/roundcell and pleomorphic tumors, respond to doxorubicin on gene expression level. In most cases, this response seems to be

### Table 2: Summary of the genes that were up-regulated by doxorubicin treatment, including the log ratios

| Samples with increased expression (n) | Gene symbol | Mean log ratio | Range of log ratio | Samples with decreased expression (n) | Mean log ratio (Range of) log ratio |
|-----------------------------------|-------------|----------------|-------------------|--------------------------------------|-----------------------------------|
| 15                  | TNFRSF10B  | 1.96           | 0.41/4.49         | 0                                   | -                                 |
| 12                  | CDKN1A     | 1.85           | 0.59/4.10         | 1                                   | -0.99                             |
| 12                  | GADD45A    | 1.44           | 0.58/2.88         | 1                                   | -0.59                             |
| 12                  | FAS        | 1.36           | 0.27/3.87         | 1                                   | -0.28                             |
| 12                  | CD40       | 0.94           | 0.39/2.11         | 0                                   | -                                 |
| 11                  | PAWR       | 1.16           | 0.33/2.51         | 1                                   | -1.25                             |
| 11                  | TNFAIP3    | 2.16           | 0.23/3.67         | 0                                   | -                                 |
| 10                  | NFKBIA     | 2.04           | 0.43/3.34         | 2                                   | -0.57                             |
| 10                  | IER3       | 3.81           | 0.71/6.08         | 2                                   | -0.60                             |
| 10                  | PSEN1      | 0.84           | 0.23/1.61         | 2                                   | -0.46                             |
| 10                  | RIPK2      | 2.02           | 0.42/3.65         | 2                                   | -0.60                             |
| 10                  | PEA15      | 0.61           | 0.23/0.96         | -1                                  | -0.68                             |
| 10                  | BCL2A1     | 2.18           | 1.03/5.08         | -1                                  | -1.12                             |
| 10                  | NGFB       | 3.06           | 0.40/5.80         | 1                                   | -0.93                             |
| 10                  | BIRC3      | 2.06           | 0.63/3.23         | 1                                   | -0.56                             |
| 10                  | CD44       | 1.57           | 0.26/3.09         | 1                                   | -0.56                             |
| 9                   | MCL1       | 1.46           | 0.67/2.08         | 3                                   | -0.65                             |
| 9                   | HSPA9      | 1.25           | 0.46/1.95         | 2                                   | -0.50                             |
| 9                   | BTG1       | 0.77           | 0.31/1.50         | 1                                   | -1.06                             |
| 9                   | HSP90B1    | 0.80           | 0.21/1.10         | 0                                   | -                                 |
| 9                   | SQSTM1     | 2.03           | 0.27/3.23         | 0                                   | -                                 |
| 9                   | PPP1R1SA   | 3.16           | 0.65/4.40         | 0                                   | -                                 |
| 9                   | IRF1       | 1.38           | 0.60/2.94         | 0                                   | -                                 |
| 9                   | CYCS       | 0.82           | 0.34/1.37         | 0                                   | -                                 |
| 9                   | MDM2       | 1.76           | 0.33/3.58         | 0                                   | -                                 |

Negative log ratios stand for down-regulated genes.
Table 3: Summary of the genes that were down-regulated by doxorubicin treatment, including the log ratios

| Samples with decreased expression (n) | Gene symbol | Mean log ratio | Range of log ratio | Samples with increased expression (n) | Mean log ratio | Range of log ratio |
|--------------------------------------|-------------|----------------|-------------------|---------------------------------------|----------------|-------------------|
| 11                                   | CD14        | -2.99          | -0.27/-5.38       | 0                                     | -              | -                 |
| 10                                   | TIA1        | -0.68          | -0.24/-1.05       | 1                                     | -              | 0.77              |
| 10                                   | YWHAH       | -0.72          | -0.27/-1.80       | 0                                     | -              | -                 |
| 10                                   | PPP3CA      | -0.69          | -0.34/-1.14       | 0                                     | -              | -                 |
| 10                                   | ITGB2       | -2.87          | -0.58/-5.11       | 0                                     | -              | -                 |
| 9                                    | PDGFRA      | -1.32          | -0.42/-3.30       | 2                                     | 2.62           | 1.21/4.03         |
| 9                                    | RASA1       | -0.93          | -0.42/-1.17       | 0                                     | -              | -                 |
| 8                                    | LDHB        | -0.60          | -0.32/-1.25       | 2                                     | 1.11           | 0.64/1.58         |
| 8                                    | CSF1R       | -3.05          | -0.63/-5.08       | 0                                     | -              | -                 |
| 8                                    | RARA        | -0.95          | -0.51/-1.23       | 0                                     | -              | -                 |
| 7                                    | ANXA4       | -1.10          | -0.64/-1.16       | 4                                     | 1.37           | 0.84/2.33         |
| 7                                    | CTSB        | -0.93          | -0.23/-1.97       | 3                                     | 0.94           | 0.82/1.13         |
| 7                                    | DAPK1       | -1.35          | -0.59/-2.07       | 2                                     | 1.40           | 1.19/1.60         |
| 7                                    | CASP1       | -1.69          | -0.71/-2.90       | 2                                     | 1.44           | 0.97/1.90         |
| 7                                    | PRF1        | -1.68          | -0.54/-3.68       | 2                                     | 0.79           | 0.67/0.91         |
| 7                                    | EPHB4       | -1.30          | -0.68/-2.34       | 1                                     | -              | 0.30              |
| 7                                    | HCK         | -1.79          | -0.39/-3.65       | 1                                     | -              | 1.08              |
| 7                                    | ESD         | -0.76          | -0.44/-0.95       | 1                                     | -              | 0.36              |
| 7                                    | DHCR24      | -1.97          | -0.75/-4.34       | 0                                     | -              | -                 |
| 7                                    | PPP1CB      | -0.99          | -0.62/-1.56       | 0                                     | -              | -                 |
| 7                                    | MYO18A/TIAF | -1.23          | -0.67/-2.02       | 0                                     | -              | -                 |
| 7                                    | BIRC5       | -2.95          | -1.17/-4.91       | 0                                     | -              | -                 |
| 7                                    | PDGFRB      | -1.28          | -0.61/-2.26       | 0                                     | -              | -                 |
| 7                                    | ATG5        | -1.39          | -0.61/-3.19       | 0                                     | -              | -                 |
| 7                                    | DOCK1       | -0.86          | -0.40/-1.23       | 0                                     | -              | -                 |
| 7                                    | PTPN13      | -1.97          | -0.69/-3.17       | 0                                     | -              | -                 |
| 7                                    | BTK         | -1.78          | -0.39/-3.28       | 0                                     | -              | -                 |
| 7                                    | SYK         | -4.26          | -0.34/-8.06       | 0                                     | -              | -                 |

Positive log ratios stand for up-regulated genes.
Figure 4
Heatmaps of expression changes of selected genes associated with apoptotic pathways after incubation with doxorubicin for 24 h ordered by responder group (5 a,b), grading (6 a,b), and histologic subtype (7 a/b). Expression changes as determined by comparison analysis were considered only if the probeset showed at least one P detection in untreated/treated sample pairs. Excluded expression changes are shown in grey. Only probesets with expression changes in 7 or more samples are given. Horizontal rows represent individual probesets. Vertical columns represent individual samples (left to right: low responders to high responders); gene symbols are indicated to the right. Gene expression changes are indicated by a continuous scale; the brightest red indicating ≥ 4-fold up-regulation; and, brightest green ≥ 4-fold down-regulated gene expression relative to the untreated control sample.
Table 5: Summary of genes beyond the ones already mentioned in the text that were found to be up-regulated by doxorubicin treatment in more than 50% of the probes.

| Gene symbol | Gene name                                                                 | Probes upregulated | Additional information                                                                                                                                                                                                 | Apoptotic function               |
|-------------|---------------------------------------------------------------------------|--------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------|
| PAWR        | PRKC (protein kinase C) apoptosis WT1 (Wilms tumor gene) regulator protein | 11                 | STS with high WT1 mRNA expression levels have poorer outcome than those with low levels [89,90]. Ecteinascidin that has been shown to be effective against STS also increases expression of PAWR [92]. PAWR inhibits the PKC (atypical protein kinase)-NF-(kappa)B (nuclear factor-(kappa)B)-XIAP pathway [91]. | proapoptotic                      |
| TNFAIP3     | tumor necrosis factor, alpha-induced protein 3                            | 11                 | TNFAIP3 down-regulates the TNF-α-induced NFκB signalling pathway [93,94].                                                                                                                                              | antiapoptotic                     |
| NFKBIA      | nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha | 10                 | The doxorubicin analog DA-125 reduces proliferation in HT1080 fibrosarcoma cells through a NFκB dependent pathway [96,97]. MDM2 (upregulated in 9 samples in our series) is overexpressed in several liposarcoma subtypes [58] and increases NFκB activity in a p53 dependent manner and thereby leads to doxorubicin resistance [98]. | proapoptotic/antiapoptotic        |
| IER3        | immediate early response 3                                                | 10                 | IER3 function is increased by p53, that is induced by doxorubicin. IER3 is involved in cell cycle arrest and programmed cell death [99,100].                                                                 | proapoptotic                      |
| PSEN1       | presenilin 1                                                              | 10                 | Effects are mediated via Bcl-2 interaction [101].                                                                                                                                                                        | proapoptotic                      |
| RIPK2       | receptor-interacting serine-threonine kinase 2                           | 10                 | RIPK2 is cell death inducing and NFκB activating, via caspase 1 activation [102,103].                                                                                                                                   | proapoptotic                      |
| PEA15       | phosphoprotein enriched in astrocytes                                     | 15                 | RPEA15 is regulating caspase-3 function in epidermal cells [104], but has not yet been associated with doxorubicin treatment or apoptosis in sarcoma cells.                                                                   | antiapoptotic                     |
| BCL2A1      | BCL2 (B-cell CLL/lymphoma 2)-related protein A1                          | 10                 | BCL2A1 stabilizes the mitochondrial membrane [105,106].                                                                                                                                                                  | antiapoptotic                     |
| NGFB        | nerve growth factor, beta polypeptide                                    | 10                 | NGF reduces apoptosis induced by chemotherapeutics in sarcoma cells [109].                                                                                                                                              | antiapoptotic                     |
| BIRC3       | baculoviral IAP repeat-containing 3                                       | 10                 | BIRC3 is associated with chemotherapy resistance in Ewing sarcoma, rhabdomyosarcoma [110] and prostatic cancer [111].                                                                                                   | antiapoptotic                     |
| CD44        | cell surface glycoprotein CD44                                            | 10                 | CD44 is a proapoptotic factor in FAS mediated apoptosis in sarcoma cells [112], but is also connected to cancer drug resistance [113]. CD44 has successfully been used as a target for liposomal encapsulated doxorubicin [114]. | proapoptotic/antiapoptotic        |
Table 4: Pearson coefficient calculated for the candidate genes describing the correlation of the gene expression changes measured microarray and rTPCR.

| Gene symbol | Pearson coefficient |
|-------------|---------------------|
| CD40        | 0.945               |
| CDKN1A      | 1.000               |
| FAS         | 0.813               |
| GADD45A     | 0.848               |
| ITGB2       | 0.999               |
| NFKBIA      | 0.882               |
| PAWR        | 0.913               |
| PPP3CA      | 0.638               |
| TNFAIP3     | 0.997               |
| TNFRSF10B   | 0.998               |
| YWHAH       | 0.370               |

based on an increase of the extrinsic pathway such as TRAIL Receptor 2 and FAS but also members of the intrinsic pathway such as BCL2A1 were found to be differentially expressed. Interestingly several factors (NFKBIA, GADD45A, RIPK2, and PAWR) point to the NFKB transcriptional factor as possible mediator of doxorubicin effects.

Our results indicate that gene expression profiling may be a promising approach to improve the understanding of the diverse modes of programmed cell death in liposarcoma following doxorubicin treatment and can provide a molecular basis for new chemotherapeutic strategies.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
AD developed the study design, coordinated the work, interpreted the data and prepared the manuscript. LKH carried out and interpreted the microarrays. AMC carried out statistical analyses, have given substantial contribution to conception and design as well manuscript preparation. OM improved the study design and corrected the manuscript. HJ prepared the figures and gathered patients data. HHH was helpful in preparing the manuscript and conceived the work. ML carried out cell culture and developed the idea, study design and conceived the work.

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