

Prognostic significance of drug-regulated genes in high-grade osteosarcoma

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About 25–45% of patients with high-grade osteosarcoma poorly respond to chemotherapy with an increased risk of relapse and the development of metastasis. Therefore, the aim of this study was the evaluation of the prognostic value of eight previously identified drug-regulated candidate genes on osteosarcoma therapy outcome. Gene expression of 8 candidate genes was analyzed in 35 formalin-fixed, paraffin-embedded, laser-microdissected osteosarcoma biopsies. The prognostic value of these genes was evaluated by the correlation of gene expression with therapy outcome, overall survival and event-free survival in univariate and multivariate analysis. Upon univariate analysis, the expression of *MALAT-1*, *IMPDH2*, *FTL* and *RHOA* significantly correlated with response to chemotherapy. Expression of all four genes was increased in the poor responder group. Upon multivariate analysis, *IMPDH2* maintained its independent prognostic value ($P=0.025$). Concerning the overall survival of the patients, we observed a significant association with the expression of *FTL*, *PHB*, *ATAD2*, *ACTN1* and *RRM2* as well as lactate dehydrogenase serum levels. In the subgroups of patients with high expression of these genes and those with elevated lactate dehydrogenase levels, the mean overall survival was decreased 1.7-, 1.9-, 2.2-, 2.4-, 1.5- and 4.5-fold, respectively. Except *RRM2*, all genes and lactate dehydrogenase serum levels remained significant in the multivariate analysis. In addition, the event-free survival was significantly decreased in the subgroups of patients with high *FTL*, *ATAD2* and *IMPDH2* expression (1.8-, 6.3- and 2.4-fold, respectively). These data demonstrate that among the identified genes are valuable markers for the prediction of osteosarcoma therapy outcome. Especially *IMPDH2* and *FTL* are promising candidates for the stratification of osteosarcoma patients into low- and high-risk groups. Owing to their involvement in drug action these genes may further be potential targets for the modulation of drug sensitivity.

Modern Pathology (2007) 20, 1085–1094; doi:10.1038/modpathol.3800937; published online 27 July 2007

Keywords: chemotherapy; gene expression; laser microdissection; osteosarcoma; prognosis

Osteosarcoma is the most common primary tumor of the bone, typically affecting the long tubular bones of children and adolescents. The prognosis of high-grade osteosarcoma treated with surgery alone has been very poor, with 5-year survival rates below 20%.¹ Major advances in treatment over the last three decades, in particular the introduction of neoadjuvant chemotherapy markedly improved the outcome with long-term relapse-free survival rates ranging from 55 to 75%.^{2,3} However, the remainder of patients poorly respond to chemotherapy with an

increased risk of relapse and the development of metastasis. Therefore, a need exists to stratify patients at diagnosis into low- and high-risk groups improving the outcome of high-risk patients and minimizing the toxicity of therapy for low-risk patients by means of a risk-adapted therapy. Several parameters have already been investigated for their prognostic significance, including morphological and biochemical markers like tumor volume, size and localization, serum levels of lactate dehydrogenase and alkaline phosphatase and proteins involved in multidrug resistance like P-glycoprotein.^{4–8} However, besides the presence of metastases at the time of diagnosis the histological response to chemotherapy is currently the strongest prognostic factor in high-grade osteosarcoma.⁹ As the extent of necrosis can only be assessed after several weeks of therapy this indicator is unsuitable for an early risk

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Received 2 May 2007; revised 12 June 2007; accepted 18 June 2007; published online 27 July 2007

stratification. Therefore, reliable factors which can be determined at the time of diagnosis to allow the prediction of therapy outcome still need to be identified. On one hand, the knowledge of these factors could be used to identify high-risk patients before the onset of therapy and on the other, these factors may facilitate the development of new therapeutic strategies. In this respect, especially genetic markers have been under investigation in the past few years.

Cytogenetic analysis of osteosarcomas yielded a large number and variety of karyotypic alterations.¹⁰ Some of these chromosomal rearrangements could be associated with altered therapy outcome. For example, high frequency of allelic imbalance at locus 4q12 containing the oncogene *c-kit*, a tyrosine kinase receptor, has been observed in osteosarcoma patients, which was concordant with *c-kit* overexpression. Normal status at this locus significantly correlated with better overall survival.¹¹ Furthermore, patients with a copy number increase at 8q have been shown to have a statistically significant poor distant disease-free survival.¹² As genetic alterations on the chromosomal level will have substantial impact on gene expression, also specific genes have been investigated for their association with therapy outcome. Characterization of the expression of ephrins, the eph receptor protein tyrosine kinase-specific ligands, revealed a distinct expression profile in osteosarcoma tissues and cell lines. Within this protein family, a correlation with poor clinical prognosis could be demonstrated for the expression of *ephrin-B1*.¹³ In addition, a correlation with poor response to chemotherapy could be shown for osteosarcomas overexpressing the transcription factor *c-fos*.¹⁴ A large set of genes differentially expressed in osteosarcoma samples with good response to chemotherapy compared to those with poor response have been identified in osteosarcoma biopsies using expression profiling by microarrays. These data suggest a specific expression signature of osteosarcomas with poor response covering genes involved mainly in growth control, extracellular matrix remodelling, osteoclastogenesis, tumor progression, multidrug resistance and detoxification.^{15–17} Together, these studies demonstrate that gene expression analysis is a very promising approach for the identification of reliable prognostic factors.

In this study, we investigated the mRNA expression profile of eight drug-regulated genes, previously identified by subtractive hybridization of cDNA derived from drug-treated osteosarcoma cell lines vs cDNA from untreated control cells.¹⁸ We assumed that genes involved in drug action are highly enriched for potential predictive markers. To evaluate their prognostic potential, the expression of these candidate genes was analyzed in osteosarcoma biopsy samples and correlated with clinical parameters like tumor necrosis in response to chemotherapy, the overall survival and the event-free survival. To overcome the known problem

of cellular heterogeneity in tumor samples, laser microdissection was used to isolate the tumor cells from paraffin sections before gene expression analysis.

Materials and methods

Patients

Thirty-five high-grade osteosarcoma patients were included in this study. Formalin-fixed paraffin-embedded specimens, collected by open biopsy before chemotherapy were retrieved from the archive of the Institute of Osteopathology of the University Hospital Hamburg-Eppendorf. All tumors were diagnosed between 1996 and 2005 and treated according to the standard treatment protocol (COSS96) of the Cooperative Osteosarcoma Study (COSS). The mean follow-up was 3.4 years ranging from 0.3 to 8.5 years. Twenty-two patients were continuously disease free, seven died of the disease and six were alive with metastases. There was no loss to follow-up. To demonstrate that this study group is representative, known clinical parameters were compared to a control group ($n = 50$) diagnosed during the same period and treated with the same chemotherapy protocol.

Response to preoperative chemotherapy was assessed histologically by the pathologist according to the six-grade scale of Salzer-Kuntschik *et al*¹⁹ where grade 1 denotes no viable tumor cells; grade 2 denotes solitary viable cells or one islet of less than 0.5 cm; grade 3 denotes less than 10%; grade 4 denotes 10–50%; grade 5 denotes more than 50% and grade 6 denotes no effect of chemotherapy. A good response was defined as less than 10% viable tumor cells corresponding to response grades 1–3. According to this categorization, gene-expression data from 18 good and 17 poor responders were included in this study. Beside the expression of drug-regulated candidate genes, serum levels of alkaline phosphatase and lactate dehydrogenase have also been included in this study. Elevated levels of alkaline phosphatase were defined as >300 U/l (males and females <13 years), >187 U/l (females, 13–17 years), >390 U/l (males, 13–17 years), >104 U/l (females >17 years) and >129 U/l (males >17 years). Elevated lactate dehydrogenase levels were defined as >300 U/l (males and females <15 years), >225 U/l (males >15 years) and >214 U/l (females >15 years). This study has been approved by the Ethical Committee of the University of Heidelberg, Germany (no: 315/2004).

Microdissection

Laser microdissection was carried out as described previously.²⁰ In brief, 10 μ m tissue sections were mounted on 0.17 mm glass slides (PALM Microlaser Technologies AG, Bernried, Germany) covered with

a 1.35 μm thin polyethylene-naphthalene membrane (PALM). Sections were deparaffinized in two changes of XEM-200 (Vogel GmbH, Giessen, Germany) for 2 min, re-hydrated in 100% ethanol, 96% ethanol and 70% ethanol, for 1 min each and briefly washed in distilled water (Invitrogen, Karlsruhe, Germany). Sections were stained for 1 min in Mayer's hematoxylin solution (Sigma, Deisenhofen, Germany), blued in 0.025% sodium bicarbonate (Sigma) for 2 min, washed in distilled water (Invitrogen), counterstained for 2 min in eosin Y solution (Sigma), washed again in distilled water and air dried for 30 min.

Microdissection was carried out using the Micro-Beam system from PALM Microlaser Technologies. Isolated cells were digested in 40 μl proteinase K digestion buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0, 2% SDS) containing 1 μl PCR-grade proteinase K (14–22 mg/ml, Roche, Mannheim, Germany) at 56°C for 16 h.

RNA Extraction and Reverse Transcription

Total RNA was extracted using the Absolutely RNATM Nanoprep kit (Stratagene, Amsterdam, Netherlands). This kit allows rapid purification of high-quality RNA from extremely small samples like cells harvested by laser microdissection. The included DNase digestion removes contaminating genomic DNA, which would disturb downstream quantification analysis. Lysis buffer (100 μl) was added to the proteinase K digestion mixture and extraction of RNA was carried out according to the manufacturer's protocol. Finally, RNA was eluted in 15 μl elution buffer.

Reverse transcription was carried out using 1 μl SensiscriptTM reverse transcriptase (Qiagen, Hilden, Germany), 0.5 mM dNTPs, 250 ng random primer (Invitrogen), 10 U RNase inhibitor (Invitrogen) and 13.5 μl RNA in a total volume of 20 μl . The reaction was carried out at 37°C for 2 h.

Preamplification and Real-Time Quantitative PCR

A standard PCR was used for preamplification of the target sequences. To minimize DNA cross-contamination and contamination of samples by PCR product carryover an additional DNase digestion step was performed. The PCR mix containing 0.2 mM dNTPs, 1.5 mM MgCl₂, 2 μl 10 \times PCR buffer and 0.5 μl DNaseI (Stratagene) in a total volume of 18.25 μl was incubated for 20 min at 37°C in a thermal cycler. DNaseI was inactivated by heating to 94°C for 10 min before 1 μl cDNA, 0.25 μl of each primer and 0.25 μl Platinum Taq polymerase (Invitrogen) were added. Samples were heated to 94°C for 3 min followed by 15 cycles of denaturation at 94°C for 20 s, annealing at 58°C for 30 s and extension at 72°C for 45 s and a final extension step at 72°C for 7 min. To avoid false-positive amplification of

genomic DNA, primers were designed to span at least two exons.

Quantitative real-time PCR was performed in a LightCycler instrument (Roche Diagnostics) in a total volume of 20 μl using the LightCycler FastStart DNA Master^{Plus} SYBR Green I kit (Roche Diagnostics) and 2 μl of the PCR product as template. Samples were heated to 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 2 s, annealing at 58°C for 7 s and extension at 72°C for 14 s. After the last cycle, a melting-curve analysis was performed to verify the specificity of the amplified PCR products.

The amount of PCR product was calculated using an external standard curve and LightCycle software. Calculated gene expressions were normalized on the basis of the porphobilinogen deaminase (*PBGD*) expression in the corresponding samples.

Statistics

Descriptive statistics were performed for demographic and clinical characteristics separately. Categorical and continuous data including absolute and relative numbers were expressed as percentages or median (range), respectively. For statistical analyses, response to chemotherapy was dichotomized (ie, good responder <10% viable tumor cells vs poor responder >10% viable tumor cells). For comparison regarding gene expression in these subgroups, the Mann-Whitney *U*-test was performed. Univariate and multivariate logistic regression analysis was performed to identify the predictive potential of eight candidate genes on response to chemotherapy. Since, we used an exploratory approach, any prognostic factor with a $P < 0.1$ was included in the multivariate analysis. Risk ratios were estimated and given with 95% CI. Observed overall and event-free survival estimates were based on Kaplan-Meier survival curves together with 95% CI. All calculations for significance were two-sided and were performed at the 5% level.

Univariate and multivariate Cox-regression analysis was performed to identify factors predictive of overall- and event-free survival. Overall survival was calculated from the date of diagnostic biopsy until death from any cause or event-free survival from date of diagnostic biopsy until diagnosis of distant metastasis or death, whichever occurred first. The log-rank test was used to compare survival curves. To discriminate between low and high-gene expression level, the mean expression levels of all analyzed samples were used as cutoff.

The statistical software used for calculation was SPSS 14.0 for Windows (SPSS Inc., Chicago, USA).

Results

Thirty-five high-grade osteosarcoma patients diagnosed between 1996 and 2005 were included in this

study. Criteria for inclusion were a radiological and histological confirmed diagnosis of high-grade osteosarcoma, a complete follow-up, known serum levels of alkaline phosphatase and lactate dehydrogenase and the availability of sufficient biopsy material suitable for laser microdissection. To demonstrate that this study group is representative, known clinical parameters were compared to a control group ($n=50$) diagnosed during the same period. No significant differences could be detected be-

tween the study group and the control group (Table 1).

The eight candidate genes analyzed in this study have recently been identified and described as differentially expressed in the osteosarcoma cell lines Saos-2, HOS and MG 63 after treatment with the cytotoxic drugs methotrexate, cisplatin and doxorubicin, respectively.¹⁸ Referring to these results, drug-treatment induced a more than two-fold change in gene expression of *RHOA*, *FTL* (ferritin

Table 1 Data of analyzed high-grade osteosarcoma patients and the control group

| Data | Study group | | Control group | | P-value |
|----------------------------|-------------|-----------------|---------------|-----------------|---------|
| | Value | No. of patients | Value | No. of patients | |
| <i>Age (years)</i> | | | | | |
| Median | 19 | | 21 | | 0.649 |
| Range | 8–51 | | 7–66 | | |
| <i>Sex</i> | | | | | |
| Female | | 16 | | 19 | 0.151 |
| Male | | 19 | | 31 | |
| <i>Histologic response</i> | | | | | |
| Good | | 18 | | 27 | 0.654 |
| Poor | | 19 | | 23 | |
| <i>Survival</i> | | | | | |
| Alive | | 28 | | 43 | 0.810 |
| Dead | | 7 | | 7 | |
| Mean (months) | 78 | | 101 | | 0.810 |
| Range (months) | 6–102 | | 3–110 | | |
| <i>Localization</i> | | | | | |
| Femur proximal | | 5 | | 25 | 0.472 |
| Femur distal | | 16 | | | |
| Tibia proximal | | 8 | | 19 | 0.472 |
| Humerus proximal | | 3 | | 3 | |
| Pelvis | | 1 | | 3 | |
| NA | | 2 | | 0 | |
| <i>Subtype</i> | | | | | |
| Osteoblastic | | 18 | | 45 | 0.319 |
| Chondroblastic | | 5 | | | |
| Fibroblastic | | 2 | | | 0.319 |
| Telangiectatic | | 4 | | 5 | |
| NA | | 6 | | 0 | |
| <i>Size</i> | | | | | |
| Mean (cm) | 11.3 | | 9.5 | | 0.372 |
| Range (cm) | 4.2–13.5 | | 3.7–15.0 | | |
| <i>Staging^a</i> | | | | | |
| Stage II | | 32 | | 50 | 0.372 |
| Stage III | | 3 | | 0 | |
| <i>Resection</i> | | | | | |
| R0 | | 35 | | 48 | 0.522 |
| R1 | | 0 | | 2 | |
| R2 | | 0 | | 0 | |
| <i>Follow-up</i> | | | | | |
| Mean (years) | 3.4 | | 4.5 | | 0.243 |
| Range (years) | 0.3–8.5 | | 0.6–9.2 | | |

NA, not available.

^aAccording to the Enneking staging system.

light chain), *IMPDH2* (inosine-monophosphate dehydrogenase II), *PHB* (prohibitin), *ACTN1* (α -actinin), *RRM2* (ribonucleotide reductase subunit M2), *ATAD2* (ATPase family, AAA domain containing 2) and *MALAT-1* (Metastases associated in lung adenocarcinoma transcript 1). In addition to these eight candidate genes, serum alkaline phosphatase and serum lactate dehydrogenase levels, which have already been reported to be of prognostic value were included in this analysis.

Upon univariate analysis, the expression of *RhoA*, *FTL*, *IMPDH2* and *MALAT-1* was significantly associated with response to chemotherapy (Table 2). The expression of these genes was significantly increased in the poor-responder group. The mean expression of *MALAT-1* was increased 5.97-fold ($P=0.001$) the expression of *IMPDH2* was increased 2.32-fold ($P=0.001$), the expression of *RHOA* was increased 2.12-fold ($P=0.026$) and *FTL* expression was 2.74-fold higher in the poor responder group compared to the good responders ($P=0.009$)

(Figure 1). No significant correlation of gene expression and therapy outcome could be detected for the other genes analyzed, namely *PHB*, *ACTN1*, *ATAD2* and *RRM2* and for alkaline phosphatase and lactate dehydrogenase serum levels, tumor size, age, subtype and the stage of the disease.

The prognostic value of univariate significant genes *RhoA*, *FTL*, *IMPDH2* and *MALAT-1* was then further evaluated by multivariate analysis. Because some studies already reported a prognostic significance of alkaline phosphatase and lactate dehydrogenase serum levels, these parameters have also been included, although not significant in the univariate analysis. Upon multivariate analysis *IMPDH2* expression remained significant and turned out to be an independent predictor of therapy outcome (Table 3).

In 33 cases, clinical follow-up data were available and used to evaluate the impact of gene expression in the tumor biopsy on overall and event-free survival. The mean expression levels of all analyzed

Table 2 Univariate logistic regression analysis of the impact of candidate gene expression on response to chemotherapy

| Gene | Regression coefficientB | Exp(B) | 95% confidence interval | P-value |
|----------------|-------------------------|--------|-------------------------|---------|
| <i>RhoA</i> | 0.056 | 1.057 | 1.000–1.117 | 0.049 |
| <i>FTL</i> | 0.001 | 1.001 | 1.000–1.002 | 0.057 |
| <i>IMPDH2</i> | 4.752 | 115.86 | 3.233–4151.6 | 0.009 |
| <i>MALAT-1</i> | 0.003 | 1.003 | 1.001–1.005 | 0.012 |

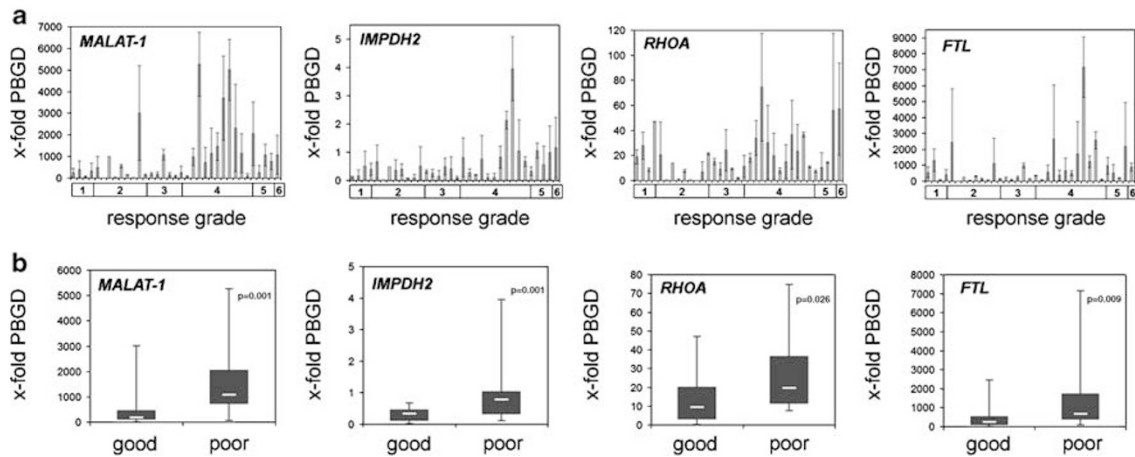


Figure 1 (a) Gene expression levels of *MALAT-1*, *IMPDH2*, *RHOA* and *FTL* in osteosarcoma biopsies. Two independent measurements have been done for each tissue section. Values are expressed as x-fold expression compared to the housekeeping genes porphobilinogen deaminase (PBGD) and presented as mean \pm s.d. (b) Gene expression compared to therapy outcome. The white lines indicate the medians and the boundaries of the boxes the 25th and 75th percent percentile. The whiskers indicate the highest and lowest values. *P*-values were determined by Mann–Whitney *U*-test.

Table 3 Multivariate logistic regression analysis of the impact of candidate gene expression on response to chemotherapy

| Gene | Regression coefficientB | Exp(B) | 95% confidence interval | P-value |
|---------------|-------------------------|--------|-------------------------|---------|
| <i>IMPDH2</i> | 4.209 | 67.297 | 1.715–2640.4 | 0.025 |

samples were used to discriminate between low and high expression. Kaplan–Meier analysis revealed a strong association of high *FTL*, *PHB*, *ACTN1*, *ATAD2* and *RRM2* expression with poor overall survival. In addition, elevated lactate dehydrogenase serum levels were significantly associated with poor overall survival (Figure 2). The mean overall survival rates were significantly reduced in the subgroups with high expression of these genes and elevated lactate dehydrogenase levels, respectively. Except *RRM2*, all factors remained significant upon multivariate analysis (Table 4).

Beside their association with overall survival, high expression of *FTL* and *ATAD2* were also associated with a lower probability of event-free survival. Further, the mean event-free survival of patients with high expression of *IMPDH2* was

significantly decreased (Figure 3). The mean event-free survival rates in the subgroups with high expression of *FTL*, *ATAD2* and *IMPDH2* were decreased 1.8-, 6.3- and 2.4-fold, respectively. Upon multivariate analysis, *ATAD2* and *IMPDH2* remained significant (Table 5).

Discussion

Despite the significant improvement of the long-term survival rates of osteosarcoma patients by the introduction of neoadjuvant chemotherapy, about 30–40% of patients will relapse. These patients and those with initial metastases have a very poor prognosis, with approximately 20% long-term survival. Therefore, the identification of prognostic

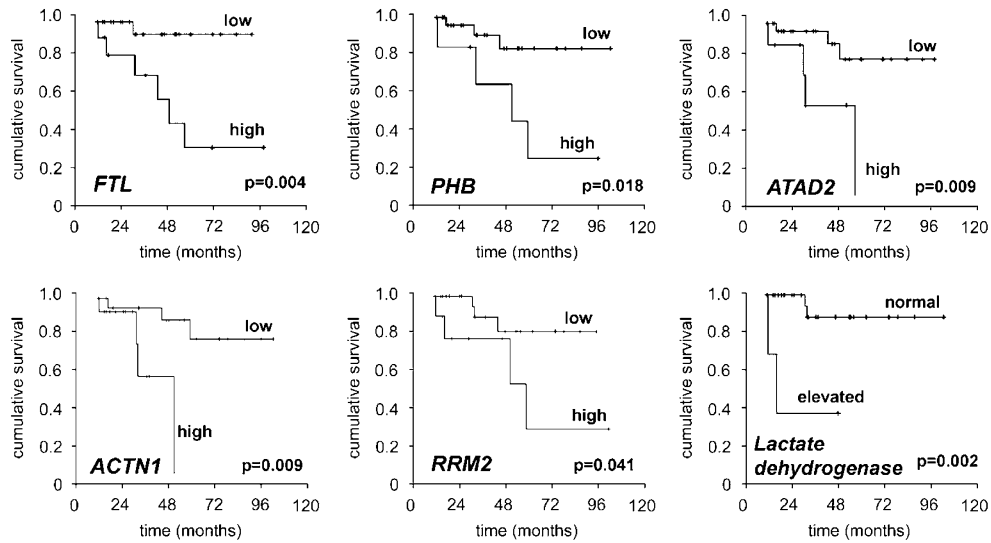


Figure 2 Kaplan–Meier analysis of overall survival in the subgroups of osteosarcoma patients with low and high expression of *FTL*, *PHB*, *ATAD2*, *ACTN1* and *RRM2* and normal and elevated serum levels of lactate dehydrogenase, respectively. *P*-values were determined by log-rank test. Genes without significant association to overall survival are not shown.

Table 4 Mean overall survival in subgroups with low/high expression of *FTL*, *PHB*, *ATAD2*, *ACTN1* and *RRM2* and normal/elevated serum levels of lactate dehydrogenase

| Prognostic factor | Level | No. of patients | Mean survival (months) | 95% CI survival | Exp(B) | 95% CI Exp(B) | P-value | |
|-------------------|----------|-----------------|------------------------|-----------------|--------|---------------|------------|--------------|
| | | | | | | | Univariate | Multivariate |
| <i>FTL</i> | Low | 22 | 90.13 | 80.88–99.37 | 11.60 | 1.39–96.42 | 0.004 | 0.045 |
| | High | 11 | 53.02 | 30.31–75.73 | | | | |
| <i>PHB</i> | Low | 27 | 89.06 | 75.75–102.3 | 5.12 | 1.14–23.0 | 0.018 | 0.040 |
| | High | 6 | 48.11 | 23.08–73.15 | | | | |
| <i>ATAD2</i> | Low | 25 | 88.42 | 74.67–102.1 | 5.90 | 1.29–26.86 | 0.009 | 0.008 |
| | High | 8 | 39.69 | 23.48–55.89 | | | | |
| <i>ACTN1</i> | Low | 20 | 87.66 | 73.22–102.09 | 9.28 | 1.48–58.65 | 0.007 | 0.007 |
| | High | 13 | 36.67 | 25.83–47.51 | | | | |
| <i>RRM2</i> | Low | 24 | 82.65 | 69.99–95.30 | 4.23 | 0.94–19.04 | 0.041 | 0.307 |
| | High | 9 | 54.24 | 27.36–81.12 | | | | |
| LDH | Normal | 25 | 92.49 | 80.36–104.6 | 12.06 | 1.66–87.24 | 0.002 | 0.028 |
| | Elevated | 3 | 20.57 | 1.28–39.85 | | | | |

CI, confidence interval; LDH, lactate dehydrogenase.

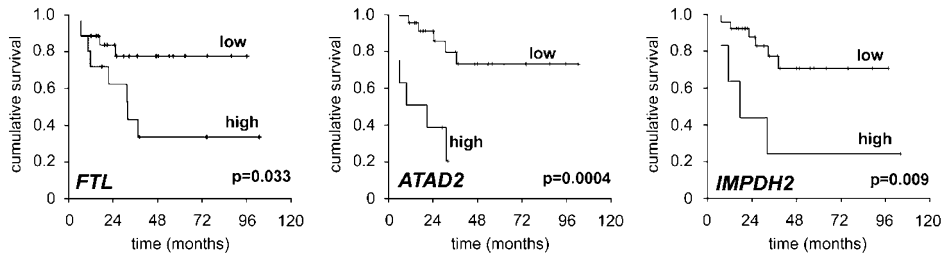


Figure 3 Kaplan–Meier analysis of event-free survival in the subgroups of osteosarcoma patients with low and high expression of *FTL*, *ATAD2* and *IMPDH2*. *P*-values were determined by log-rank test. Genes without significant association to event-free survival are not shown.

Table 5 Event-free survival in subgroups with low / high expression of *FTL*, *ATAD2* and *IMPDH2*

| Prognostic factor | Level | No. of patients | Mean survival (months) | 95% CI survival | Exp(B) | 95% CI Exp(B) | P-value | |
|-------------------|-------|-----------------|------------------------|-----------------|--------|---------------|------------|--------------|
| | | | | | | | Univariate | Multivariate |
| <i>FTL</i> | Low | 22 | 76.93 | 60.99–92.86 | 3.49 | 1.02–11.94 | 0.033 | 0.440 |
| | High | 11 | 43.27 | 18.13–68.42 | | | | |
| <i>ATAD2</i> | Low | 25 | 80.04 | 63.33–96.76 | 7.29 | 2.04–26.14 | 0.0004 | 0.001 |
| | High | 8 | 12.78 | 4.28–21.27 | | | | |
| <i>IMPDH2</i> | Low | 28 | 69.51 | 53.58–85.45 | 4.47 | 1.29–15.43 | 0.009 | 0.017 |
| | High | 5 | 28.70 | 0.00–61.77 | | | | |

CI, confidence interval.

factors is still of great clinical importance to stratify patients into high- and low-risk groups and for the development of new risk-adapted treatment strategies. We hypothesized that genes involved in drug action are especially qualified for the prediction of therapy outcome. Thus, our approach to the identification of such prognostic factors was based on the pre-selection of candidate marker genes by subtractive hybridization of drug-treated vs -untreated osteosarcoma cell lines.¹⁸ The prognostic potential of the identified drug-regulated genes should be evaluated in this study. As the cellular heterogeneity is a major disadvantage in the analysis of tumor tissues, paraffin sections were used for the isolation of RNA. To minimize the influence of non-malignant bystander cells, a minimal amount of 95% tumor cells was defined as threshold for analysis. If the amount of tumor cells was less than 95%, laser microdissection was used for their isolation. Reliability and reproducibility of laser microdissection and the subsequent steps of gene-expression analysis have already been demonstrated.²⁰ Because serum levels of lactate dehydrogenase and alkaline phosphatase have already been reported to be of prognostic value in several studies^{2,5,21} these factors have been integrated in the analysis.

Our results showed that high expression of four analyzed genes was associated with a poor response to chemotherapy. The most powerful factor predicting therapy outcome in our study was *IMPDH2* (inosine-monophosphate dehydrogenase II). Upon multivariate analysis *IMPDH2* expression maintained its independent prognostic power, suggesting

that *IMPDH2* might be considered for the stratification of osteosarcoma patients into low- and high-risk groups. *IMPDH* encodes the rate-limiting enzyme in *de novo* guanine nucleotide biosynthesis, maintaining cellular guanine deoxy- and ribonucleotide pools needed for DNA and RNA synthesis. Two isoforms of *IMPDH* have been described. Type I is constitutively expressed in normal cells, whereas Type II activity has been shown to be increased in malignant cells.^{22,23} Thus, *IMPDH* has been considered as an attractive target for cancer therapy and *IMPDH* inhibitors like tiazofurin and benzamide riboside have been successfully applied in clinical trials.^{24,25} In these studies, *IMPDH* inhibition exhibited a good clinical response in patients with acute myeloid leukemia and chronic myeloid leukemia in blast crisis. The responses correlated with a decline in *IMPDH* activity and GTP concentration and were related to the level of the NAD analogue formed in the leukemic cells. In multiple myeloma cells inhibition of *IMPDH* has been shown to induce caspase-dependent apoptosis and cell-cycle arrest.²⁶ Further, increased *IMPDH* mRNA levels have been detected in methotrexate (MTX)-treated and MTX-resistant human colon cancer and erythroleukemia cells. The authors suggested that the increased *IMPDH* expression might be interpreted as a compensation for the inhibition of dihydrofolate reductase by MTX. Inhibition of *IMPDH* significantly increased the sensitivity of the resistant cell lines to MTX, indicating that targeting of *IMPDH* might be a promising approach to minimize the development of resistance.^{27,28} Besides our finding of the prognostic

significance of *IMPDH2* gene expression, these data suggest an additional role of *IMPDH2* as a promising target for the modulation of drug resistance in osteosarcomas.

Although the expression of *MALAT-1*, *RhoA* and *FTL* did not remain statistically significant in multivariate analysis, which might be due to the limited number of analyzed tumor samples, their expression was significantly elevated in osteosarcoma patient with poor response to chemotherapy.

We initially identified a sequence termed *Pro1859* as an upregulated gene in cisplatin-treated osteosarcoma cell lines. However, sequence alignments and northern blot analysis turned out that this sequence is obviously part of a much longer transcript, recently identified as *MALAT-1* (metastasis associated in lung adenocarcinoma transcript 1).²⁹ *MALAT-1* is widely expressed in normal tissue and interestingly represents a putative noncoding RNA. It was identified by subtractive hybridization of primary non-small cell lung tumors that either did or did not metastasize. The observed association of *MALAT-1* expression in osteosarcoma biopsies with therapy outcome suggests a crucial role of this transcript in the pathology of different kinds of tumors, including osteosarcomas. In addition, these data support the growing evidence that noncoding RNAs may play key roles in a variety of fundamental cellular processes.^{30,31}

The third gene that was associated with poor response to chemotherapy in our study was *RHOA*. Rho GTPases form a subgroup of the Ras superfamily of GTP-binding proteins that are involved in a wide spectrum of cellular processes including cell morphology, regulation of cell migration through the re-organization of the actin skeleton, transcriptional regulation, apoptosis and malignant transformation. Overexpression of *RHOA* has been reported in different kinds of tumors including breast, lung and colon carcinomas.³² Furthermore, *RHOA* expression levels have been shown to correlate with tumor stage in testicular germ cell tumors³³ and with invasion and metastasis in bladder cancer.³⁴ Thus, Rho GTPases are supposed to be a promising cellular target for novel tumor therapies.^{35,36} Inhibition of Rho-GTPase activity has been linked to the inhibition of cell proliferation and invasiveness, the induction of apoptosis, and the enhancement of chemosensitivity.^{37–39} Accordingly, we observed a negative correlation of *RHOA* expression and therapy outcome, suggesting an involvement of *RHOA* in the modulation of drug sensitivity also in osteosarcomas.

The fourth gene that we found to be associated with response to chemotherapy was *FTL* (ferritin light chain). Together with ferritin heavy chain molecules, these proteins form the ferritins, which are important regulators of the intracellular iron content. Depletion of intracellular iron has been shown to induce major cellular alterations including cell-cycle arrest and apoptosis.⁴⁰ Likewise, down-

regulation of *FTL* by antisense oligonucleotides has been shown to inhibit growth and to induce apoptosis in breast carcinoma cells⁴¹ and to enhance sensitivity to oxidative stress and to apoptosis in a melanoma cell line.⁴² So far, *FTL* overexpression has been reported in several malignancies including colon and breast cancer as well as in a cell line derived from a metastatic lymph node.^{43,44} Further, low levels of ferritin light chain have been shown to be associated with a good response in breast cancer.⁴⁵ In agreement with these data, we observed a significantly higher expression of *FTL* in osteosarcoma biopsies from patients with poor response to chemotherapy and consequently an association of *FTL* expression with therapy outcome.

Interestingly, besides the response to chemotherapy, *FTL* expression was also associated with the overall- and event-free survival of osteosarcoma patients in this study. As *FTL* expression is tightly coupled to cell-cycle progression and growth control as well as the chemoinvasive properties of tumor cells,⁴² these data suggest that high expression of *FTL* may characterize a more aggressive type of malignancy with an increased proliferation capacity.

Beside *FTL*, also *PHB*, *ACTN1* and *ATAD2* turned out to be associated with overall survival of osteosarcoma patients. These genes are less associated with drug response and resistance rather than cellular motility (*PHB*),⁴⁶ reorganization of the cytoskeleton (*ACTN1*)⁴⁷ and chaperone-like functions (*PHB* and *ATAD2*).⁴⁸ In addition, we confirmed the prognostic significance of serum lactate dehydrogenase concerning overall survival.

In summary, we could confirm our hypothesis, that among drug-regulated genes are valuable candidates for osteosarcoma prognosis. Some of the evaluated genes have already been shown to be involved in the development and progression of different kinds of malignancies. Our data suggest that these genes may also be relevant for osteosarcoma prognosis. Especially, the highly significant association of *IMPDH2* expression with response to chemotherapy indicates that *IMPDH2* is a promising candidate for the stratification of osteosarcoma patients into low- and high-risk groups. Concerning survival, *FTL* and *ATAD2* expression turned out to be the most powerful predictors associated with overall- and event-free survival. In addition to the observed prognostic value, the involvement in drug action indicates that the identified genes might also be promising targets for the modulation of drug sensitivity and the development of new therapeutic strategies.

Acknowledgements

This study was supported by a grant from the 'Deutsche Krebshilfe e.V.' and the EuroBoNeT. The Department of Experimental Orthopedics, Orthopedic University Hospital Heidelberg is partner of

the EuroBoNeT consortium, a European Commission-granted Network of Excellence for studying the pathology and genetics of bone tumors. We thank A-M Eschlbeck and A-M Heuser for technical assistance.

Disclosure/conflict of interest

There is no conflict of interest to declare.

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