

Resveratrol Induces Apoptosis and Alters Gene Expression in Human Fibrosarcoma Cells

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Abstract. *Background/Aim: Metastatic fibrosarcomas still represent a therapeutic dilemma. Commonly used chemotherapeutic agents such as doxorubicin have been proven effective in fewer than 30% of all cases disseminated of fibrosarcoma. Elderly patients with cardiac disease are not suitable for systemic chemotherapy with doxorubicin. We therefore tested the apoptotic effects of the natural and well-tolerated compound resveratrol on human fibrosarcoma cells (HT1080). Materials and Methods: Vital, apoptotic and necrotic cells were quantified using flow cytometric analysis. Gene expression was analyzed by RNA microarrays. Results: Application of resveratrol induced apoptotic cell death and significantly reduced proliferation of HT1080 cells. Correspondingly, expression of apoptosis-associated genes was altered in microarray analysis. Conclusion: This in vitro study demonstrates the anticancer activity of resveratrol against human fibrosarcoma cells. These results provide experimental support for in vivo trials assessing the effect of the natural polyphenol resveratrol.*

Soft tissue sarcomas are a heterogeneous group of malignant neoplasias which represent about 1% of all new cancer cases in Europe and the United States (1). Fibrosarcomas are rare soft tissue sarcomas originating from the intra- and intermuscular fibrous tissues, fascia and tendons and account for approximately 3% of all soft tissue sarcomas. Therapy

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for fibrosarcomas should be individualized and multimodal. The therapy of choice involves surgical resection with a wide margin of healthy tissues, usually followed by radiation treatment in order to decrease local recurrence (2, 3). Unfortunately, about 50% of all patients develop distant metastases and are ineligible for surgical treatment (4, 5). In cases of advanced metastatic disease, the median survival time with and without chemotherapy treatment is less than 12 months (6, 7). Few agents, such as doxorubicin, ifosfamide and dacarbazine, have been proven effective in the therapy of soft tissue sarcomas (3). However, the results of these treatments are poor and often exhibit no significant improvements in overall survival (8). Doxorubicin, which has been the most frequently used chemotherapeutic agent in the treatment of soft tissue sarcomas, demonstrates response rates of 20 to 30% in patients with disseminated disease (9, 10). The combination of doxorubicin with ifosfamide is more effective, leading to higher response rates than doxorubicin alone, but is associated with severe short- and long-term toxicities, including cardiomyopathy and bone marrow suppression (11-13). The recently published EORTC 62012 trial which involved 455 patients with locally advanced, unresectable or metastatic high-grade soft-tissue sarcomas concluded that an intensified therapy with doxorubicin and ifosfamide is not suitable for palliation of advanced soft-tissue sarcomas because of the severe side-effects and should only be used when the specific goal is tumor shrinkage (13). Furthermore, the utility of the first-line cytostatic doxorubicin is limited by dose-related and cumulative myocardial toxicity, especially in elderly patients with pre-existing cardiac disease (14). However, age is an important determinant of sarcoma occurrence and the incidence of soft-tissue sarcomas increases dramatically at ages of 50 years and above which are naturally associated with higher prevalence of cardiac diseases (15). To date, there are no effective and well-tolerated cytostatics for the palliative

treatment of patients who are not suitable for aggressive anthracycline-based chemotherapy. Hence, there is still a need for alternative and well-tolerated compounds that exhibit anti-neoplastic effects against sarcoma cells.

Within the scope of this trial, we investigated the effects of the natural compound resveratrol on human fibrosarcoma cells. Resveratrol is a natural polyphenole (trans-3,5,4-trihydroxy-stilbene) that occurs in red grapes, peanuts and various berries. The highest naturally occurring levels of resveratrol are found in *Polygonum cuspidatum*, a plant which has been used in traditional Asian medicine to treat inflammation and other ailments (16). Resveratrol is also found at high concentrations in red wine and might account for the reduced risk of cardiovascular disease in the French population ('French Paradox') despite their high intake of saturated fats because of the antioxidant properties of resveratrol (17). Since resveratrol is very well-tolerated and a naturally occurring compound, it has been highly studied for the treatment of many diseases, including cancer (18, 19). Several *in vitro* and *in vivo* studies demonstrated the anticancer activity of resveratrol in a wide range of solid malignancies, such as skin, breast, colon and prostate cancer (20-24). Resveratrol interfered with all three major stages of tumorigenesis and induced apoptosis of cancer cells *via* different mechanisms (20). These mechanisms included the inhibition of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- κ B) by enhancing the activity of the NF- κ B inhibitor inhibitor of kappa B (7). Recent oncological studies have suggested that the constitutive activation of NF κ B is associated with poor prognosis and resistance to chemotherapy in many types of human cancer, including sarcomas (25-27). More specifically, it has been reported that the tumor invasiveness of fibrosarcoma cells can be significantly reduced by inhibiting NF κ B activity (28-30).

In other malignant cell lines, resveratrol has also been shown to alter the activity of cyclo-oxygenase 2, cyclin-dependent kinase 2 and silent information regulator 1, which are known to induce apoptosis of human fibrosarcoma cells (31-33). Further apoptosis-inducing mechanisms in cancer cells included the modulation of p53 and the activation of apoptosis-inducing-fragment-ligand-mediated apoptosis (34, 35).

Inspired by these findings, we examined in the following study the apoptosis-inducing activity of resveratrol against human fibrosarcoma cells and analyzed subsequent gene alterations after treatment with resveratrol *in vitro*.

Materials and Methods

Cell line. Human fibrosarcoma cells, HT1080, were purchased from the American Type Culture Collection (ATCC, cell line CCI 121; Wesel, Germany) and maintained in modified Eagle's medium (MEM) and non-essential amino acids with 10% fetal bovine serum

supplemented with 1% penicillin (100 U/ml) and streptomycin (100 μ g/ml), 1% sodium pyruvate and 1% L-glutamine. The cells were cultured in a humidified atmosphere at 37°C with 5% CO₂ in 25 cm² flasks.

Reagents. Resveratrol was obtained from Cayman Chemical (Ann Arbor, MI, USA) and was dissolved in distilled water to obtain a concentration of 2,195 ng/ml. This is the highest achievable concentration in human plasma after oral administration (36).

Cell treatment. For each drug experiment, 80 μ l of cell suspension (3 \times 10⁶ cells/ml) were placed in 6-well plates containing the medium. After 24 h, the medium was replaced and the drugs were added to each well. The cultivated human fibrosarcoma cells were incubated with resveratrol or povidone iodide as control. Different incubation times were chosen to identify the possible time dependency of the effects. All experiments were repeated for each of three consecutive passages.

Flow cytometric analysis. After incubation, the floating cells were collected together with the supernatant and adherent cells, which were harvested by trypsinisation. The cells were pelleted by centrifugation, resuspended in 195 μ l binding buffer (Bender MedSystems, Vienna, Austria) and incubated with 5 μ l annexin V (BD Biosciences, Heidelberg, Germany) and 10 μ l propidium iodide (PI) (Bender MedSystems) following the manufacturer's instructions. The cells were analyzed immediately using a FACS flow cytometer (FACS Calibur; BD Biosciences). For each measurement, 20,000 cells were counted. Dot plots and histograms were analyzed using CellQuest Pro software (BD Biosciences). Annexin V binds phosphatidylserine on the outer membranes of cells, which then becomes exposed on the surface of apoptotic cells. Thus, the annexin V-positive cells are considered apoptotic. PI is an intercalating agent that cannot permeate through the cell membranes of viable or early apoptotic cells. Therefore, PI stains only the DNA of necrotic or very late apoptotic cells. In this study, annexin V- and PI-positive cells were termed necrotic. Annexin V- and PI-negative cells were counted as viable.

Cell morphology. The morphology of the adherent and suspended cells was examined and documented using a phase-contrast Zeiss Axiovert 25 microscope (Carl Zeiss, Jena, Germany).

Oligonucleotide microarray analysis. To identify the changes in gene expression caused by treatment with resveratrol, total RNA was purified from cells after incubation with resveratrol for 6 h using a RNeasy KIT from Qiagen (Hilden, Germany) as specified by the manufacturer. RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). For microarray analyses, we applied the methods previously described by Daigeler *et al.* (37). We used the Affymetrix GeneChip platform, employing a standard protocol for sample preparation and microarray hybridization. A one-way ANOVA model followed by Tukey's Honestly Significant Difference test was used to verify the hypothesis that there were no differences in expression between the drug-treated group and the control group. Multiplicity correction was performed using Benjamini and Hochberg procedure to control the false discovery rate (FDR) at 0.05%. In a pair-wise comparison of the differentially expressed genes between the control and the resveratrol-treated cells

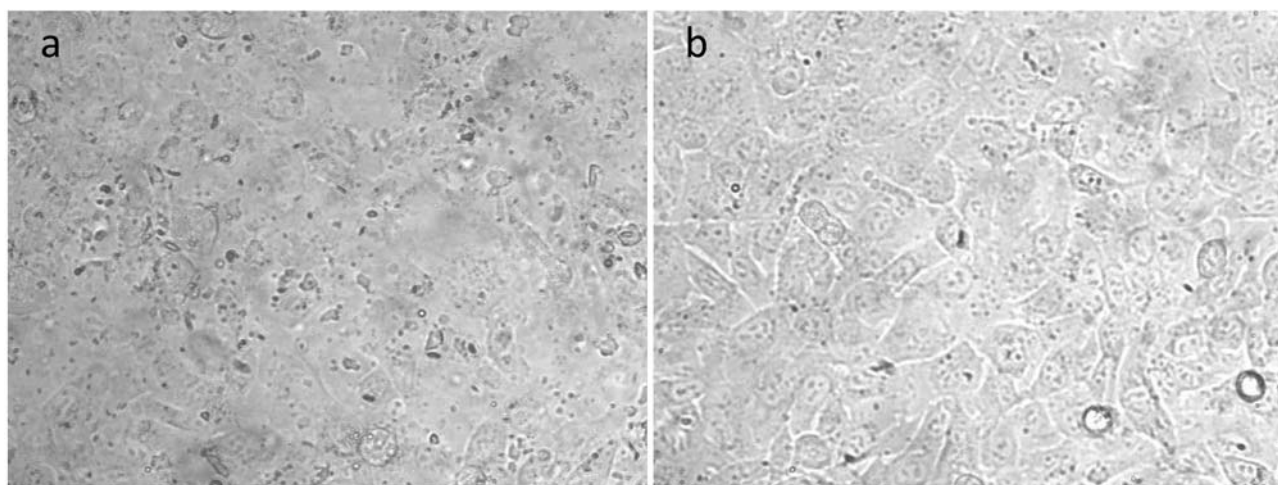


Figure 1. Morphological changes after treatment. Representative photographs demonstrating morphological changes in HT1080 cells induced by resveratrol (a) following 24 h of treatment compared with untreated control cells (b).

identified by the ANOVA analyses, a subset of genes was identified that displayed a conjoint regulation in treated cells. Genes were placed in this latter group if they exhibited a mean increase or decrease of twofold or more compared to the control cells. This subset of genes was subjected to GeneTrail (38) software to identify any over-representation of genes associated with the regulatory pathways of the Kyoto Encyclopaedia of Genes and Genomes (KEGG) and TRANSPATH databases. Microarray data were deposited in the GEO public database (accession number: GSE59704). These methods fulfilled the Minimal Information About A Microarray Experiment criteria (<http://www.mged.org/miame>).

Statistical analysis. The results of FACS analysis were used to determine the percentages of viable, apoptotic and necrotic cells, which are expressed as the means \pm SD from at least three independent experiments and consecutive passages. In this study, comparisons between the experimental groups (resveratrol-treated versus control) were performed using one-way measures of variance (one-way ANOVAs) over all time points (Tukey test). Results were considered statistically significant for p -values ≤ 0.05 .

Results

Resveratrol induced apoptosis of HT1080 fibrosarcoma cells.

The first moderate but significant apoptotic response was observed after 6 h of incubation; the percentage of viable cells was reduced to 79.75 \pm 0.3% (mean \pm SD, $p < 0.001$) compared to 94.17 \pm 1.54% in the control group. Here, exposure to resveratrol led to a marked increase in the number of apoptotic cells, whereas the number of necrotic cells increased only moderately: 12.88 \pm 1.09% of the cells were detected to be apoptotic and 6.97 \pm 1.16% necrotic. In the control group only 2.87 \pm 1.06% were found to be apoptotic and 2.29 \pm 0.46% necrotic. The highest apoptosis rates were reached after 24 h treatment, reducing the

population of the viable cells significantly to 76.99 \pm 1.38%, whereas 14.23 \pm 3.99% were observed to be apoptotic and 7.89 \pm 2.43% necrotic. In contrast, the viability of the control cells after 24 h was 94.88 \pm 1.44%, with only 3.39 \pm 1.43% apoptotic and 2.34 \pm 0.76% necrotic.

Addition of resveratrol induced morphological changes and cell detachment. As demonstrated in Figure 1, resveratrol reduced the cell density of HT1080 fibrosarcoma cells, indicating decreased rates of cell division and proliferation. Furthermore, such treatment led to shrinkage of cells and dissolution of confluent cell groups, followed by complete cell detachment. Longer incubation with resveratrol resulted in obvious morphological aberrations.

Microarray analysis revealed differential gene expression patterns of HT1080 cells treated with resveratrol. Based on comparison analysis algorithm, resveratrol remarkably altered the expression of different combinations of probe sets. In resveratrol-treated cells, microarray analyses identified noticeable expression changes in 1,318 genes. Out of these, 71.93% (n=948) were down-regulated and 28.07% (n=370) up-regulated.

To obtain an overview of the biological processes affected by resveratrol, we analyzed lists of regulated targets of the pathways that were over-represented in our data set using the GeneTrail application (38). Significant over-representation was detected in several pathway categories that included apoptosis, cell cycle, pathways in cancer, p53 signaling, cytoskeleton and cell adhesion (Table I). To understand the molecular details underlying the diverse modes of cell death in fibrosarcoma cells, we focused on the differentially expressed apoptosis-associated genes that were altered by resveratrol (Figure 2, Table II).

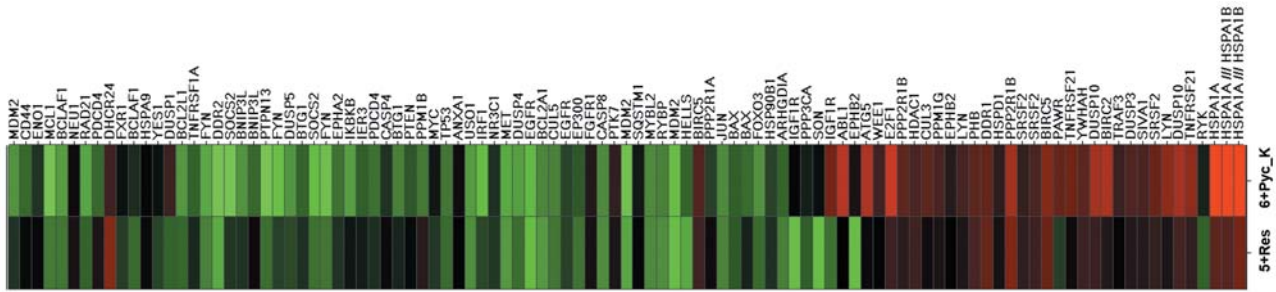


Figure 2. Hierarchical clustering: Overall gene expression pattern of reliably measured probe sets. Horizontal rows represent individual probe sets/genes; vertical columns represent individual samples. 5+Res: Resveratrol; 6+Pyc_K: Untreated control. Color scale: black, mean expression (indicates unchanged expression), brightest green, 0.25×mean value (indicates expression level below the mean), brightest red, 4×mean value (indicates expression level higher than the mean).

Table I. Microarray analysis using GeneTrail software identified significant changes in the following computed KEGG pathway categories with *p*-values below 0.05 and false discovery rate (FDR) adjustments (Benjamini-Hochberg). The genes in the sub-categories were significantly up- or down-regulated by 2-fold or more in the treatment group compared to the control group.

Significant subcategories ($\alpha=0.05$, FDR-adjusted)	<i>p</i> -Value	Expected number of regulated genes	Observed number of regulated genes
Apoptosis	0.018	61	84
Cell cycle	0.017	9	21
Pathways in cancer	0.017	20	35
P53 signaling	0.016	5	13
Cytoskeleton	2.8e-3	68	99
Cell adhesion	0.014	33	51
Metabolic pathways	0.039	65	47

Discussion

Fibrosarcomas are rare tumours within the heterogeneous group of soft tissue sarcomas and respond poorly to conventional treatments, such as chemotherapy and radiation. Despite excellent rates of local disease control, treatment options in distant metastatic disease, especially in pulmonary locations, are very limited and have an associated median survival of less than 12 months (6, 7). Due to the rarity of fibrosarcomas, the development of new therapeutics has been difficult, and the lack of novel chemotherapy protocols remains a major problem. Additionally, elderly patients with cardiac subdisease are ineligible for doxorubicin-based chemotherapy, which is still considered first-line treatment at metastatic disease stage. For these reasons, there has been increasing interest in assessing whether the cardiotoxicity of doxorubicin could be mitigated by antioxidant compounds. Recently, the natural compound resveratrol was found to protect cardiomyocytes against doxorubicin-induced free radicals, thus attenuating cardiotoxicity due to doxorubicin in mice (39, 40). Interestingly, resveratrol also has anticancer activity against a

wide range of solid malignancies (20, 41). Because resveratrol is extremely well-tolerated and no severe side-effects have been reported, it is categorized as a nutritional supplement in most European countries and is readily available. The numerous advantages of resveratrol inspired us to analyze its anticancer activity in human fibrosarcoma cells.

In our study, resveratrol significantly induced apoptosis of HT1080 cells. Moreover, it led to decreased cell division and distinct morphological changes. To further elucidate the actions of resveratrol on a molecular basis, we analyzed changes in expression of apoptosis-related genes using microarray technology. The most notable gene alterations were found in members of the (phosphatidylinositol-4,5-bisphosphate) PI3K/V-akt murine thymoma viral oncogene (AKT) signaling pathway (Table II). Interestingly, the PI3K/AKT pathway is widely dysregulated in many solid malignancies, including several soft-tissue sarcoma subtypes, and many studies have shown this pathway to be vital to the growth and survival of cancer cells (42-45). Here, multiple mechanisms have been found to induce PI3K/AKT signaling, such as activating mutations of key

Table II. Summary of the expression changes of apoptosis-related genes for resveratrol-treated cells, compared to untreated cells.

GeneID	Gene name	Oncological relevance (ref)	Signal log ratio
<i>MALAT1</i>	Metastasis-associated lung adenocarcinoma transcript 1	Overexpressed in a wide range human carcinomas and undifferentiated embryonal sarcoma cells (59)	-1.9
<i>NFAT5</i>	Nuclear factor of activated T-cells 5	Promotes proliferation and migration of renal and colon carcinoma cells (16, 43)	-1.4
<i>NFEL2L</i>	Nuclear factor (erythroid-derived 2)-like 2	Enhances the survival of soft-tissue sarcoma cells (rhabdomyosarcoma, leiomyosarcoma, synovial sarcoma, liposarcoma) (36)	-1.2
<i>WASF2</i>	Wiskott-Aldrich syndrome protein family member 2	Associated with poor survival prognosis in patients with breast and hepatocellular carcinoma (34, 85)	-1.2
<i>IGFR1</i>	Insulin-like growth factor receptor 1	Overexpressed on various types of tumor cells and contributes to tumor cell growth (28, 65)	-1.1
<i>TFRC</i>	Transferrin receptor protein 1	Expressed in more than 80% of malignancies in public databases and linked to angiogenesis in solid tumors (24)	-1.0
<i>ARGHDIA</i>	Rho GDP dissociation inhibitor alpha	Overexpressed in chemoresistant fibrosarcoma and colon carcinoma cells (72)	-0.9
<i>DDR2</i>	Discoidin domain receptor tyrosine kinase 2	Drives growth and survival of soft-tissue sarcoma cells (rhabdomyosarcoma, leiomyosarcoma, fibrosarcoma) (6)	-0.9
<i>JAK1</i>	Janus kinase 1	Inactivation of JAK1 in fibrosarcoma cells leads to loss of invasion <i>in vitro</i> and metastasis <i>in vivo</i> (77)	-0.8
<i>PIK3CB</i>	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit beta	Required for growth of phosphatase and tensin homolog-deficient coloncarcinoma cells (82)	-0.8
<i>MDM2</i>	Mouse double minute 2 homolog	Overexpression in fibrosarcoma and amplified in liposarcoma (37, 81)	-0.8
<i>EGFR</i>	Epidermal growth factor receptor	Overexpressed in a variety of soft-tissue sarcomas and often associated with chemoresistance and a poor clinical outcome (70)	-0.8
<i>AKT3</i>	V-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)	Contributes to invasive migration and tumor metastasis in various malignancies (18)	-0.7
<i>CBL</i>	Cbl proto-oncogene, E3	Positive regulator of the PI3K/AKT signaling pathway in cancer cells (75).	-0.6
<i>BRCA2</i>	ubiquitin protein ligase Breast cancer 2, early onset	Enhances osteosarcoma cell proliferation and survival (68)	-0.5
<i>KRAS</i>	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	Mutation associated with breast cancer and radiation-induced sarcomas (39)	-0.5
<i>IRS2</i>	Insulin receptor substrate 2	Overexpression promotes progression of metastatic fibrosarcoma <i>in vivo</i> (3)	-0.5
<i>CASP2</i>	Caspase 2, apoptosis-related cysteine peptidase	Promotes invasion of carcinoma cells by activating PI3K/AKT signaling pathway (69)	-0.5
<i>COX3</i>	Cytochrome c oxidase III	Involved in TRAIL-induced apoptosis in rhabdomyosarcoma and fibrosarcoma cells (42, 58)	+0.3
		Decreased expression is associated with apoptosis resistance in colon cancer cells (55)	+1.0

+, Increased expression; -, decreased expression; The number following the treatment slot is the fold over-/underexpression compared to the untreated cells.

genes such as *PI3KCB* and *AKT3* (46). However, the PI3K pathway can also be activated by upstream receptors or regulators such as insulin-like growth factor receptor 1 (*IGFR1*), insulin receptor substrate 2 (*IRS2*) and Cbl proto-oncogene (*CBL*) (47-49). In the current study, resveratrol led to a significant down-regulation of *IGFR1*, *IRS2*, *CBL*, *PI3KCB* and *AKT3* suggesting that the PI3K/AKT signaling pathway may play a role in apoptosis induction in human fibrosarcoma (Table II). The only experimental study assessing the impact of PI3K/AKT pathway in fibrosarcoma cells demonstrated that inhibition of PI3K *via* small molecular inhibitors remarkably reduced the invasive potential of HT1080 cells *in vitro* (50). However, the role of

the PI3K/AKT pathway in human fibrosarcoma is still unknown and warrants further research because the novel and well-tolerated group of PI3K inhibitors could be potentially useful therapeutic options.

Interestingly, we found a correlation between apoptotic efficacy of resveratrol and down-regulation of the oncogene mouse double minute 2 homolog (*MDM2*). Overexpression of *MDM2* in HT1080 fibrosarcoma cells has already been described but its function is still unknown (51). *MDM2* gene amplification is found in nearly all well-differentiated and dedifferentiated liposarcomas (52). Currently, the *MDM2* inhibitor RG7112 could pass a proof-of-mechanism study in patients with well-differentiated liposarcomas successfully

reducing tumor proliferation (53). MDM2 overexpression is also associated with aggressive leiomyosarcoma, undifferentiated pleomorphic sarcoma, spindle-cell sarcoma and adult fibrosarcoma, as well as having involvement in tumor progression in a wide range of soft-tissue malignancies (54-56). Considering that down-regulation of *MDM2* was associated with increased apoptosis in our study, MDM2 antagonists may be attractive therapeutic agents for human fibrosarcomas.

Resveratrol also led to a down-regulation of Janus kinase 1 (*JAK1*) and epidermal growth factor receptor 1 (*EGFR1*). The JAK/STAT signaling pathway is a key signal transduction pathway implicated in the pathogenesis of many types of human cancer, including several soft-tissue sarcoma subtypes (57, 58). Constitutive JAK/STAT activity has been demonstrated to cause tumorigenic inflammation and increased proliferation in a wide range of malignant diseases, including malignant fibrous histiocytoma (59-61). In fibrosarcoma, inactivation of *JAK1* led to loss of invasion *in vitro* and metastasis *in vivo* (62). Recently, pharmacological inhibition of *JAK1* was shown to induce apoptosis of rhabdomyosarcoma cells *in vivo* (58). *EGFR1*, as another interesting pharmacological target, was overexpressed in a variety of soft-tissue sarcomas. It was associated with chemoresistance and a poor clinical outcome (63). In fibrosarcoma cell lines, *EGFR1* knockdown using short interfering RNA resulted in decreased expression of matrix metalloproteinases and decreased cellular invasion (64).

Understanding the complex role of *JAK1* and *EGFR1* in sarcoma cell death may provide new opportunities for rational pathway-based therapies and drug development. Several novel JAK/STAT and *EGFR1* inhibitors have been tested in clinical trials and could be promising agents in the therapy of metastatic soft-tissue sarcomas.

In conclusion, the present *in vitro* study demonstrates that the natural compound resveratrol has the potential to induce apoptosis and alter gene expression in fibrosarcoma cells. Although a wide variety of genes and pathways were involved, the PIK3/AKT signaling pathway appears to play a key role in mediating apoptosis of HT1080 cells *via* resveratrol. Resveratrol is not meant to replace doxorubicin-based chemotherapy in patients with metastatic fibrosarcoma, but it could be a potential mild therapeutic option for patients who are not suitable candidates for chemotherapy and therefore have to undergo palliative treatment. The encouraging results of this study provide experimental support for *in vivo* trials assessing the effect of resveratrol against soft-tissue sarcomas.

Competing interests

The Authors declare that they have no competing interests.

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