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Application of metabolomics in sarcoma: from biomarkers to therapeutic targets

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1 Introduction

Sarcomas are a large and heterogeneous group of more than 70 malignant primary neoplasms derived from mesenchymal origin [1, 2]. Bone and soft tissue sarcoma are the main types of sarcoma. Although sarcoma causes approximately 1% of all cancer-related deaths, it accounts for 19–21% of cancer-related deaths in children and adolescents [2, 3]. At present, the diagnosis remains dependent on clinical description, radiographic assessment, and histopathologic staging systems in sarcoma. Despite an increased understanding of the molecular pathogenesis of sarcoma during the past two decades, reliable biomarkers to enable screening and surveillance of sarcoma are still unavailable. Importantly, identifying specific biomarkers to detect sarcoma at the onset and monitor disease progression continues to be an overwhelming challenge. Furthermore, surgery combined with neoadjuvant chemotherapy and irradiation therapy represent the current standard treatments for sarcoma, which have significantly raised the prognosis of this disease to a plateau that has been maintained for the last 30 years [2]. New therapeutic strategies as well as diagnostic, prognostic and predictive biomarkers remain unmet clinical needs for the management of sarcoma.

“Omic” technologies entail high-throughput approaches for systems-level studies of all genes or gene products, and promise a better understanding of tumor oncogenesis that can improve tumor diagnosis to facilitate the emergence of personalized tumor management [4, 5]. Although genomics, transcriptomics and proteomics have a significant impact on the general understanding of cancer-related processes, some limitations in the discovery of

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diagnostic biomarkers and treatment targets through gene/protein expression data have been recognized in recent years [6]. The major challenge is to identify the key “signals” in the data that drive traits of interest (e.g. disease, drug response, etc.), such as the genomic changes and their real influence on sarcoma phenotype. Therefore, other methods that directly affect sarcoma phenotypes, such as metabolomics have shed light on the search for diagnostic and prognostic biomarkers, and therapeutic targets.

Metabolomics is a new discipline that evaluates diverse metabolite concentrations in biological specimens to gain insight into the ongoing metabolism [7, 8]. Metabolites are highly stable end products of various metabolic pathways and are the closest link between the genotype and phenotypes of a cell. Thus metabolites may have applications as biomarkers for cancer diagnosis, prognosis, and therapeutic evaluation [9]. The number of metabolites present in a human organism is currently more than 40,000 and still expanding [10]. Metabolomics provides a comprehensive measurement of the entire metabolome that reflects the genome, epigenome, transcriptome and proteome, and their interactions with the environment [11]. Moreover, metabolomics detects an array of metabolites rather than a single metabolite in a single assay, so that the process is more convenient and the result is believed to be more indicative of sarcoma status [7]. In particular, tumor cells are known to possess unique metabolic signatures when compared with normal cells [12]. To date, metabolomic signatures have already been published from investigations aimed to discover biomarkers and treatment targets for several cancers such as colorectal, breast, liver, lung, ovarian, prostate, and pancreatic [13-19]. The integration of “omic” data reveals more than the simple sum of the individual “omic” experiments and possibly provides access to understand the interactions among all of the cellular contents [20, 21].

In this review, we summarize the current state of knowledge regarding the contribution of metabolomics to sarcomas, and its clinical applications from biomarkers to potential therapeutic targets.

2 Workflow of metabolomics

There are three major metabolomics approaches, including metabolite profiling, metabolic fingerprinting and targeted analysis (Table 1) [22-25]. Among them, metabolite profiling and metabolic fingerprinting are regarded as non-targeted analyses for all accessible metabolites in a given sample [26]. Metabolomics is a complementary analytical and high-throughput methodology for metabolite profiling that determines and quantifies known and unknown metabolites [23-25]. Metabolomic fingerprinting is the least precise approach as it enables rapid monitoring of the composition of low molecular weight compounds, without detailed identification [22-25]. Targeted analysis, as the most developed analytical approach in metabolomics, identifies and quantifies pre-defined metabolites [22-26]. The most commonly used method is metabolite profiling, allowing analysis of a given group of identified and unknown metabolites with respect to their chemical nature [22-25].

Regardless of the chosen analytical approach, there are several general steps in the metabolomics studies including: sample collection, sample preparation, metabolite extraction, and data acquisition, analysis and interpretation (Figure 1).

The first step of a metabolomics study is sample collection. Blood (serum), urine, and lysates of the cultured cells are the optimal specimens due to easy repetitive access and their high concentration of metabolites. In addition, saliva, sweat, tears, cerebrospinal fluid and other types of physiological liquids and tissues can be also used for metabolomics studies. For metabolomics studies, it is critical to preserve the collected samples as close to the in vivo state as possible [26]. For most of metabolomics analyses, the required standard sample volume is within the range of 0.1 to 0.5mL [6]. The gold standard of sample storage is freshly frozen and stored at a temperature below -80° C. However, formalin fixed paraffin-embedded (FFPE) samples at room temperature are also suitable for certain metabolomics analyses if the samples were collected properly [27].

The choice of the sample preparation method depends on the nature of the biological sample and the type of analytical technique. Quenching is a rapid procedure by which biochemical processes are stopped and the metabolism is instantly inactivated. Classic quenching methods are performed by drastically changing the temperature or pH of the sample. Perchloric acid and cold methanol are mainly used for quenching. In cell culture based metabolomics, reducing the methanol proportion in methanol/water mixtures can reduce cellular metabolite leakage.

Adequate metabolite extraction is highly important for the outcome quality of a metabolomics study. To isolate a relatively large group of metabolites, organic solvents, such as methanol, acetonitrile, chloroform, or hexane are used for extraction. For human body fluids, metabolite extraction and quenching are performed together in one step by directly adding an ice-cold methanol/water mixture to the sample. For culture cells, it was found that methanol/water mixtures were the most reproducible in terms of metabolite recovery [28]. These steps should be performed at lower temperatures (4 °C).

Despite the value of metabolomics to deliver further insight into complex phenotypes such as those observed in cancer, technologies for efficiently performing metabolomics have only emerged in recent years. The most commonly applied data acquisition methods in cancer metabolomics studies are liquid or gas chromatography (LC/GC) coupled with mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy (Table 2) [26, 29]. MS is a highly sensitive technique which allows identification and quantification of multiple metabolites. Chromatographic techniques separate a complex mixture of analyzed compounds prior to MS. NMR spectroscopy enables identification of small biomolecules based on the resonance spectra of the atomic nuclei ^1H , ^{13}C , and ^{31}P , which are commonly observed in metabolites [30-32]. Methods based on NMR can measure the liquid and solid samples at once with a minimal preparation stage. High-resolution magic angel spinning NMR (HR-MAS-NMR) spectroscopy is widely tested for metabolite-based sarcoma classification [33, 34]. More recently, direct analysis of spatial distribution of metabolites in a tissue is an emerging approach in metabolomics. Spatial distribution of metabolic markers can be analyzed both in vitro and in vivo using localized magnetic resonance spectroscopy imaging (MRSI) in sarcoma [30]. Another method for in vitro differentiation of tissue regions based on their molecular content is mass spectrometry imaging (MSI). MSI allows for high-resolution determination of spatial distribution of metabolites and lipids within the

sarcoma tissue [35]. During a typical MS or NMR measurement, mass spectra are continuously recorded resulting in a 3 dimensional dataset.

The fourth stage of metabolomics is data analysis, including several different computational and bioinformatics steps. Typically, a metabolomics data analysis consists of the following steps: noise filtering, peak detection, peak alignment, normalization, deconvolution and detection of compound spectra, mapping of deconvoluted mass spectra across several chromatograms, quantification of metabolite amounts, and statistical analysis. Subsequently, univariate statistics and multivariate statistics, including supervised and/or unsupervised methods, are used for candidate biomarker classification. Supervised methods (e.g., partial least squares-discriminant analysis (PLS-DA), orthogonal partial least-squares-discriminant analysis (OPLS-DA)) use the information of class membership to classify a given dataset. Unsupervised approaches (e.g., principal component analysis (PCA), hierarchical cluster analysis (HCA)) have been applied to investigate the innate variation in data set. Additionally, the registered molecules are identified by annotation at metabolomics databases, such as Human Metabolome Database (HMDB), METLIN, Golm database, MassBank, or LIPID MAPS [36-40].

The last stage of metabolomics is data interpretation based on pathway analysis network-based analysis and empirical correlation analysis [41].

3 Contribution of metabolomics in osteosarcoma

Osteosarcoma is the most common primary bone tumor in children and adolescents. To date, the methods for early detection of osteosarcoma are lacking. Although advances in chemotherapy have significantly increased the survival rate, recurrent and lung metastasis still occur in some patients. In addition, frequent acquisition of drug-resistant phenotypes becomes an obstacle to the chemotherapy of osteosarcoma. Research that focuses solely on an osteosarcoma patient's DNA or RNA sequence may fail to reveal functional aspects of protein activity. Metabolomics studies could directly study the phenotypes of metastasis and chemotherapy resistance for example, and may be a more reliable method to search for treatment, diagnostic, prognostic and predictive targets upon integration with genomics, proteomics and so on (Table 3).

3.1 Identification of diagnostic biomarkers and treatment targets

A better understanding of global metabolomics dysregulations in osteosarcoma is not only helping in early detection, but also in improving the clinical efficiency of osteosarcoma therapy. (Table 3)

In an independent metabolomics study, measurements were obtained for the metabolites in the serum and urine samples from three groups including osteosarcoma patients, benign bone tumor patients and healthy controls [42]. There were 260 and 180 aligned individual peaks obtained from serum and urine samples, respectively [42]. Through PCA, PLS and OPLS-DA models, the metabolomics results from the samples of osteosarcoma patients, benign bone tumor patients and healthy controls were compared [42]. 29 and 21 markedly differential metabolites in the serum and urine samples, respectively, were identified in

osteosarcoma patients[42]. Additionally, the differentially identified metabolites suggested an especially disrupted energy metabolism in osteosarcoma patients, which was characterized by significantly downregulated tricarboxylic acid (TCA) cycle, glycolysis and lipid metabolism, dysregulated sugar levels, and upregulated amino acid metabolism, glutathione metabolism and polyamine metabolism [42]. Therefore, the above metabolic profiles in serum and urine samples of osteosarcoma patients provide the potential to figure out the possible diagnostic biomarkers, and therapeutic targets in osteosarcoma patients.

Transactivation-proficient (TA) p73 α is a transcription factor belonging to the tumor suppressor gene p53 family. The intracellular pathways activated by TAp73 α were investigated via the simultaneous application of proteomics, metabolomics, and lipidomics [43]. Before “omic” analysis, the TAp73 α -induced by doxycycline and noninduced human osteosarcoma cell Saos-2 (p53 null) were successfully cultured [43]. Through proteomics analysis, differential phosphorylations present in the noninduced controls but absent in TAp73 α -induced cells were affirmed to be dependent on mTOR activation, and TAp73 α was thought to induce activation of specific phosphatases upstream of the mTOR signaling pathway [43]. Then, metabolomics analyses were carried out. The result of untargeted metabolomics showed that the metabolites could be categorized by their role in a pathway including: glycolysis, Krebs cycle, glutathione homeostasis, pentose phosphate pathway (PPP), purine metabolism, arginine-citrulline-NO metabolism, and other signaling metabolisms [43]. In glycolysis related group, the level of phosphoenolpyruvate (PEP) and lactoyl-glutathione were significantly higher in TAp73 α -induced cells when compared with that of the noninduced cells [43]. In the Krebs cycle related group, succinyl-coenzyme A (CoA), succinate, fumarate, FAD and FADH₂ were significantly accumulated in TAp73 α -induced Saos-2 cells compared with that of noninduced cells [43]. In the glutathione homeostasis related group, homocyst was significantly reduced and GSH, GSSG and adenosyl-homocysteine were significantly augmented in TAp73 α -induced cells when compared with that of noninduced cells [43]. In the pentose phosphate pathway related group, 6-phosphogluconolactone (6PGL), NADPH and NADP⁺ were significantly raised in TAp73 α -induced cells when compared with that of noninduced cells [43]. Further targeted metabolomics approach was performed to validate these results of untargeted metabolomics about NADPH [43]. In the purine metabolism related group, adenosine was increased significantly and adenine was decreased significantly in TAp73 α -induced cells when compared with that of noninduced cells [43]. In the arginine-citrulline-NO metabolism related group, accumulation of all the metabolites involved in the arginine-citrulline-NO metabolism (arginine, argino-succinate, citrulline, ornithine) was observed [43]. In the PI3K/Akt/mTOR signaling pathway related group, PIP₂ was at a significantly higher level in TAp73 α -induced cells when compared with that of noninduced cells [43]. Furthermore, through lipidomics analysis, the expression of TAp73 α might influence lipid biosynthesis and lipid oxidation to a certain extent in the absence of external stimuli [43]. In the end, the simultaneous application of multiple omics approaches, especially metabolomics analysis, profiled a holistic view of the TAp73 α downstream activated pathways, which was helpful to further understand the role TAp73 plays to mediate cell survival and promote tumor suppression signals.

Further investigation of the effect of TAp73 on metabolic profiling was done on human p53/p73 null Saos-2 osteosarcoma cell lines at early time points (8h and 16h) after the induction of TAp73 β was performed. Through metabolomics analysis, 292 biochemical compounds were detected [44]. Among the 292 compounds, 55 and 42 significantly differential compounds were found in TAp73 β -induced cells at time points 8h and 16h, respectively [44]. The TAp73-induced cells demonstrated significantly higher levels of glycolysis, amino acids and their metabolites, acetyl-CoA, polyamine, membrane phospholipids, methyl-donor S-adenosylmethionine (SAM) possibly influencing methylation and epigenetics, and arginine metabolism suggesting a role in extracellular matrix modeling [44]. Then, in order to explore the underlining gene/metabolite network of the metabolomics effects of TAp73, all the upregulated metabolites in our analysis, plus KEGG and Reactome databases were used to construct an integral gene-metabolite network related to p73 [44]. The network showed that only 2 measured metabolites (glutamine and glutamate) were unregulated and located within 2 steps from p73 [44]. Nine up-regulated metabolites were located within 4 steps, while only 7 of the not up-regulated metabolites were as close to p73 [44]. Meanwhile, the analysis demonstrated that TAp73 modulated amino acid metabolism, and glutaminolysis affected aspartate, alanine, valine, leucine and isoleucine through GLS-2 regulation [44]. Hence, through metabolomics, TAp73 was clarified to regulate multiple metabolic pathways in osteosarcoma cells. TAp73 and the related pathways could be the potential diagnostic biomarkers and therapeutic targets of osteosarcoma.

To explore the potential diagnostic biomarkers of osteosarcoma, transcriptomic and metabolomics approaches were integrated. Through transcriptomic analysis of three osteosarcoma cell lines (MG-63, Saos-2 and U-2OS) and one osteoblastic cell line, 211 significantly differentially expressed genes were identified, and 93 genes were up-regulated and 118 were down-regulated [21]. Through metabolomics analysis, 7 up-regulated and 12 down-regulated metabolites were selected by comparing the serum samples collected from 24 osteosarcoma patients with that from a control group of 32 healthy individuals [21, 42]. Then, the selected gene and metabolite signatures underwent pathway enrichment analysis according to KEGG pathway database and data integration. For gene signature, enriched pathways included: alanine, aspartate and glutamate metabolism, TCA cycle, glycolysis/gluconeogenesis, pentose phosphate pathway, pyruvate metabolism, metabolic pathways, arginine and proline metabolism, ATP-binding cassette (ABC) transporters, butanoate metabolism, glyoxylate and dicarboxylate metabolism, glycerolipid metabolism and propanoate metabolism. Enriched pathways for metabolite signatures included: purine metabolism, valine, leucine and isoleucine degradation, glycolysis/gluconeogenesis, arginine and proline metabolism, cysteine and methionine metabolism [21]. The pathway enriched in both genes and metabolites was the glycolysis pathway [21]. The genes and metabolites in the glycolysis pathway were found to be a mixed signature of four genes (ENO1, TPI1, PKG1 and LDHC) and two metabolites (lactate and pyruvate) [21]. Afterwards, an independent test dataset was constructed based on the samples from 20 osteosarcoma patients and 20 healthy control subjects, to explore the predictive value of the selected mixed metabolite and gene signature [21]. These four genes including ENO1, TPI1, PKG1 and LDHC were significantly down-regulated, and these two metabolites including lactate and

pyruvate exhibited lower levels [21]. Integrated metabolomics and transcriptomic analysis was proven to be a much more dependent, reliable and effective way to identify the true osteosarcoma samples than transcriptomic or metabolomics analysis alone [21]. These results suggest that metabolomics analysis integrated with transcriptomics analysis could be helpful to explore the diagnostic biomarker in osteosarcoma.

3.2 Identification of biomarkers and therapeutic targets for chemoresistance

The paradigm of raising chemosensitivity has been significantly revolutionized with the development of advanced “omic” technologies in the last decade [47]. Reverse chemoresistance in osteosarcoma also requires the identification of the influencing factors such as metabolites associated with chemoresistance. (Table 3)

Metabolomics was performed to explore the intracellular metabolite changes of human osteosarcoma MG-63 cells triggered by the chemotherapy agent cisplatin (CDDP) at different times of exposure [33]. The cell spectrum of control MG-63 cells in the absence of CDDP at 0 h was set as the baseline of this study [33]. The result showed that the metabolites including lipids, several amino acids and its derivatives, choline and choline-containing compounds, polyalcohols, phosphoethanolamine, nucleosides, nitrogenated bases adenosine/inosine were significantly changed [33]. Then, the cell spectra of control MG-63 cells in the absence of CDDP at 0 h and 48 h were compared as a reference for the evaluation of CDDP effects [33]. The result demonstrated that changes were found in polyalcohols, creatine, some amino acids, choline-containing compounds, nitrogenated bases adenosine/inosine and others [33]. When we compare the cell spectra between the CDDP treated cells and controls at 48 h, the metabolites including nitrogenated bases adenosine/inosine, some amino acids and other metabolites indicated alterations [33]. Further multivariate analysis of these cells revealed alterations of lipids, choline compounds, some amino acids, nitrogenated bases adenosine/inosine and others in CDDP treated cells [33]. When the spectra of CDDP treated cells at 48 h was compared with that of CDDP treated cells at an earlier time, the result showed significant changes in lipids, some amino acids, choline and more [33]. With a basis on the statistical recoupling of variables and the spectral integration of some peaks, it became apparent that the main metabolite changes were more marked at the later stage of drug exposure (24-48 h), and the most time-dependent spectral changes were related to the significant alterations in lipids, choline-containing compounds, some amino acids, polyalcohols, and nitrogenated bases adenosine/inosine [33]. Lipids, choline and choline-containing compounds were some of the main players in MG-63 cell response to CDDP, suggesting the occurrence of apoptosis and alterations in lipid metabolism regulation [33]. The decrease in glutamate and taurine possibly reflected the effects of oxidative stress overtime and activation of cell DNA-related defense mechanisms, respectively [33]. Significant reductions in polyalcohols indicated their role in cellular stress response whereas the decreasing nitrogenated bases adenosine/inosine could reflect reduced DNA synthesis [33]. To summarize, the time-dependent metabolomics changes of osteosarcoma cells treated by CDDP could help us to understand the mechanism of CDDP and find possibly predictive biomarkers of chemoresistance and therapeutic targets in osteosarcoma.

The metabolomics study was also carried out to compare the different effects of doxorubicin (DOX), methotrexate (MTX) and CDDP on human osteosarcoma MG-63 cells. The spectra of DOX-treated cells revealed marked changes in choline, glycerophosphocholine, m-inositol and others, and the spectra of MTX-treated MG-63 cells unveiled that the cellular metabolome was significantly related to lipids, phosphocholine, choline, UXP, and uridine [34]. After comparing the spectra of the cells respectively treated with CDDP, DOX and MTX, significantly different responses of choline compounds, nucleotide bases, and acetylglycosylated derivatives were observed [33, 34]. For further confirmation and research, multivariate analyses via PCA and PLS-DA model were applied. The metabolite profile of the DOX-treated cells showed residual changes in lipids; marked increases in choline and glycerophosphocholine in tandem with phosphocholine decrease; significant Gly decrease and Tyr increase accompanied by a slight decrease tendency in other amino acids; marked increase in UDP-Glc-NAC and small increases in UXP and uridine; and significant increase in creatine and decreases in GSH and m- and s-inositols [34]. The metabolite profile of the MTX-treated cells showed nearly no changes of lipids, choline, glycerophosphocholine and inositols; a large decrease in phosphocholine; increases in amino acids of Gly, Phe, Tyr and decreases in Glu and Tau; marked increase in uridine; and small decrease in creatine along with a weak decreasing tendency for GSH [34]. In comparison, the metabolite profile of the CDDP-treated cells showed significant increases in lipids content; increases in Cho and GPC and no change in PC; decreasing tendency in amino acids; changes in UXP and glucosylated derivatives; and marked decreases in m- and s-inositols, and increase in acetate(s) [34]. Furthermore, the time-dependent heat-map of effect size values was performed to evaluate several metabolites and peak ratios in cells exposed to DOX, MTX, and CDDP. In lipid metabolism, numerous marked changes were observed for CDDP response [34]. In polyunsaturated fatty acid metabolism, the increase for DOX was more evident [34]. In phospholipid related metabolism, phosphoethanolamine decreased markedly for CDDP, phosphocholine decrease markedly for MTX and DOX, and Cho and GPC increases observably for DOX [34]. In ratios heat-map, the time course increased lipid chain length and unsaturation degree in response to CDDP, decreased lipid chain length and increased some unsaturations for DOX and MTX [34]. Choline ratios were particularly sensitive markers of the cells metabolic response to DOX and MTX [34]. The amino acid metabolism could be decreased by CDDP, but increased by DOX and MTX [34]. The nucleotide base signatures could be affected by CDDP, whereas mildly increased by DOX and decreased by MTX [34]. Regarding other compounds, CDDP and DOX, but not MTX, could decreased m- and s-inositols; and acetate(s), creatine, and glutathione could be mildly increased by CDDP and decreased by DOX and MTX [34]. All in all, the significantly different effects of DOX, MTX, and CDDP on the metabolome of osteosarcoma cells provided valuable insight into the metabolic cellular responses to chemotherapy, the synergetic effects of chemotherapeutic drugs, and the potential clinical biomarkers of drug efficacy and treatment monitoring.

Although mTORC1 pathway is highly expressed in clinical osteosarcoma patients, inhibition of mTORC1 may not be enough to induce significant cell death of human osteosarcoma MG-63 cells [45]. Recently, metabolomics analysis was implemented to uncover how mTORC1 regulates osteosarcoma development [45]. The mTORC1 in osteosarcoma cells

was silenced by mTORC1 inhibitor rapamycin and Raptor silencing [45]. After comparing the metabolite profiles of mTORC1 expression cells with the mTORC1 silencing cells, there were 26 significantly differential metabolites among 189 detected metabolites [45]. Inhibition of mTORC1 led to reduced amino acid metabolites including serine and glycine in osteosarcoma cells [45]. In addition, mTORC1 regulated serine/glycine *de novo* synthesis via regulating glycolysis and serine/glycine synthesis gene expressions [45]. Through further analysis, osteosarcoma serine and glycine levels were also verified to be reduced by either rapamycin or Raptor silencing treatment [45]. Moreover, mTORC1/serine/glycine metabolic axis promoted osteosarcoma proliferation and antioxidant ability to environmental stress, resulting in cell survival [45]. Altogether, the mechanism of mTORC1-mediated serine/glycine metabolism was regarded as a significant protective system in osteosarcoma cells resisting chemotherapy, and the results might provide new predictive biomarkers of chemoresistance and novel opportunities for overcoming chemoresistance in osteosarcoma.

3.3 Identification of metabolite biomarkers for metastasis

As one of the hallmarks of osteosarcoma, lung metastasis is a complicated process whose molecular details of the metabolic transformation remain largely unknown. Characterized metabolomics changes will allow us to update viewpoints of osteosarcoma phenotypes and design new therapies towards tumor cell proliferation and migration. (Table 3)

A metabolomics study was designed to characterize the metabolic changes associated with tumor invasion and metastasis to the lungs in a mouse model with subcutaneous transplantation of murine osteosarcoma cell line LM8. In the primary tumor model, a total of 262 aligned individual peaks were obtained from serum samples [46]. Among these peaks, 52 significantly differentially expressed metabolites were identified, and were also found to be associated with the metabolism of amino acids, organic acids, fatty acids, sugars and polyols, and others[46]. To understand the metabolite changes associated with lung metastasis, the serum metabolite expression levels in the lung metastasis group were compared with those in the same group sacrificed earlier without lung metastasis [46]. Consequently, 56 serum metabolites were observed to be significantly altered [46]. Most of these metabolites were reduced during lung metastasis, however, the 2, 4-dihydroxybutanoic acid, 2-piperidinecarboxylate, palmitic acid, elaidic acid, octadecanoic acid, decosahexaenoic acid, and cholesterol were significantly raised [46]. These results illustrated that the tumor metastasis was associated with decreased carbohydrate and amino acid metabolism, but an elevated lipid metabolism. Meanwhile, several sugars and polyols, such as glucose phosphate, glucose, and gluconolactone which were all involved in the pentose phosphate pathway significantly decreased during lung metastasis; which demonstrates a special metabolic transformation through the pentose phosphate pathway as an alternative to glycolysis during tumor invasion/migration [46]. Moreover, to figure out the association between dynamic metabolic changes and osteosarcoma metastasis, the two-dimensional and three-dimensional PCA and PLS-DA score plots were constructed [46]. A total of 78 differential metabolites were identified related to the metabolism of amino acids, TCA cycle, free fatty acids, and sugars and polyols [46]. Most of them involved in glycolysis, TCA cycle and amino acids were significantly elevated before lung metastasis, revealing the high energy demand and the altered enzyme activities associated with rapid

tumor growth preparing for tumor metastasis [46]. During lung metastasis, the following findings were uncovered: cystine and 2-hydroxybutanoic acid correlated with glutathione metabolism were significantly decreased; threonic acid (metabolite of ascorbate, another cellular antioxidant as glutathione) was down-regulated, further indicating impaired antioxidant ability in the lung metastatic mice; arabitol and arabinofuranose which come from intestinal microbial degradation of nondigestible carbohydrates, were significantly reduced, which revealed the gut microbial compositional changes in the mice with lung metastasis [46]. Finally, the heat-map and the bar-plots of the differential metabolites were used to visualize the fluctuations of metabolites from osteosarcoma cell inoculation to lung metastasis [46]. Most of the metabolites were at lower levels at the beginning of cell inoculation and lung metastasis, showing a relatively lower metabolic activity during the tumor formation [46]. Most of the metabolites were at higher levels before lung metastasis, suggesting that high amount of energy and precursors were needed for cell proliferation and tumor growth because the tumor reached the largest size prior to metastasis [46]. Thus, the result of the metabolomics approach exhibited the time-dependent and characteristic changes in serum metabolites at the different stages of tumor invasion, tumor growth, and lung metastasis in a mice model. These findings will contribute to identify metabolite biomarkers with diagnostic potential for osteosarcoma and its metastasis to the lung.

4 Contribution of metabolomics in other types of sarcomas

Liposarcoma is the most common soft tissue sarcomas, accounting for roughly 20% of sarcomas. Patients with high-grade or unresectable liposarcoma have poor prognosis, although surgery and some drugs including DOX, ifosfamide, antimitotic docetaxel and antimetabolites gemcitabine seem helpful [48, 49]. Recently, a new diagnostic biomarker and treatment target of liposarcoma has been studied through metabolomics. The metabolites of three liposarcoma cell lines which often exhibit low glucose uptake by positron emission tomography (PET) were assessed [50]. Through PCA model and supervised hierarchical clustering analysis, ten metabolites, including ascorbic acid, cholesteryl sulfate, five amino acids and amino acid precursors, and three nucleosides (cytidine, thymidine, and uridine), were indicated to be consistently consumed, suggesting that liposarcoma cells had nucleoside salvage pathway activity which enables nucleoside uptake and conversion to nucleotide triphosphates that can be incorporated into DNA [50]. The nucleoside salvage pathway was found to be dependent on deoxycytidine kinase (dCK) *in vitro* and could be imaged *in vivo* by PET with 1-(2'-deoxy-2'-[18F] fluoroarabinofuranosyl) cytosine (FAC) [50]. Although liposarcoma cells exhibit nucleoside salvage activity, they are not dependent on this metabolic pathway for proliferation and survival [50]. In addition, liposarcoma cell lines and xenograft tumors were clarified sensitive to the treatment of gemcitabine which is a chemotherapeutic nucleoside analogue prodrug metabolized the same way as FAC. In other *in vitro* and *in vivo* studies, gemcitabine showed a cytotoxic effect on liposarcoma which exhibited nucleoside salvage pathway activity, and gemcitabine sensitivity was dependent on dCK expression [50]. Moreover, elevated dCK activity was evident in around 10% of liposarcoma patient samples [50]. Therefore, the liposarcoma patients with positive nucleoside salvage activity or dCK expression could be detected by PET imaging with [18F]-FAC (or an FAC derivative) and treated by gemcitabine. (Table 4)

Leiomyosarcoma and synovial sarcoma are both extremely rare forms of cancer, and represent about 5-10% of all soft tissue sarcoma respectively. Besides the difficulty of treatment, early diagnosis is also difficult. Metabolomics assessment of liposarcoma, leiomyosarcoma and synovial sarcoma was first applied in soft tissue sarcoma [27]. There were five FFPE soft tissue sarcoma tissues and five paired FFPE normal tissue [27]. 119 metabolites were robustly detected in the sarcoma samples [27]. Then through unsupervised hierarchical clustering and PCA, the differences between the paired sarcoma specimens and their normal control could be presented [27]. Eight significantly differential metabolites, including carbamoyl phosphate, CMP, ribose-phosphate, cytosine, cyclic-AMP, DL-Pipecolic acid, Ng,NG-dimethyl-L-arginine and nicotinamide were selected [27]. In addition, the pathway enrichment analysis indicated that many pathways were enriched on the basis of all 119 metabolites, including glycolysis, glutamate metabolism, the citric acid cycle, and so on [27]. Hence, the metabolomics data from FFPE soft tissue sarcoma tissues could be used as diagnostic biomarkers for soft tissue sarcoma. (Table 4)

Chondrosarcoma, the second most common primary bone malignancy in human, is a heterogeneous collection of cartilaginous-origin tumors with different outcomes depending on subtype and histological grade. Unlike many tumors, chondrosarcoma is usually resistant to chemotherapy and radiotherapy. To date, there is no report about the metabolomics study in chondrosarcoma. Based on the metabolomics study in brain cancer, 2-hydroxyglutarate (2-HG) was found as an oncometabolite was verified to be generated by mutant isocitrate dehydrogenase (IDH) [51-53]. Mutations of IDH, which is an essential enzyme for cellular respiration in the TCA cycle, have been implicated in tumorigenesis, cell maintenance, and proliferation of several diverse tumors, such as acute myeloid leukemia, angioimmunoblastic lymphomas, and glioma [54-56]. There are three subtypes of IDH, IDH1 is found in peroxisomes and cytosol, IDH2 and IDH3 are located within the mitochondria. IDH1 and IDH2 enzymes catalyze the NADP+-dependent oxidative decarboxylation of isocitrate to α -ketoglutarate (α -KG), with generation of carbon dioxide (CO₂) and NADPH [54]. Recently, cartilaginous neoplasms have been observed to significantly frequently (56%) harbor gene mutations in IDH1 and IDH2 (IDH1>IDH2) [57]. In our study, highly IDH1/2 mutations (61%) were detected in high grade chondrosarcoma with the majority (86%) of mutations being present on the IDH1 gene [58]. The IDH1/2 mutations could be a potential diagnostic biomarker for dedifferentiated chondrosarcoma [58]. Future metabolomics studies will help us to further investigate the functional roles of IDH1/2 mutations in chondrosarcoma.

5 Conclusion and future perspectives

Sarcomas are regarded to be associated with abnormal metabolic alterations which results from modifications of gene expression. Powerful technologies of metabolomics have enabled researchers to produce an unprecedented amount of data in cancers including some types of sarcomas. Both sarcoma cultured cells and sarcoma patient samples can be studied by metabolomics. Based on these metabolomics studies, distinct metabolic pathways associated with sarcoma cell proliferation, invasion/migration, drug resistance have been reported. These results may help to provide reliable diagnostic, predictive and prognostic biomarkers, and potential targets in the management of sarcoma.

Despite the promising value of initial studies, further works are necessary to pay attention to ① improving the technology of metabolomics to investigate volatile, large and specific compounds; ② establishing spectral databases of metabolites of the normal population; ③ integrating metabolomics data to genomics, transcriptomics, and proteomics results to yield better insights into the cellular mechanisms. At present, most of the metabolomics studies still remain at the exploratory stage in cancer research, including sarcoma. The future of combining phenotype, genome discovery, and bioinformatics will provide the most valuable accomplishment for metabolomics breakthrough in sarcomas.

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highlights

- Metabolomics technology is able to comprehensively determine the entire metabolome present in a sarcoma sample.
- Metabolomics studies have revealed distinct metabolic pathways associated with cell proliferation, invasion/migration, drug resistance in sarcoma.
- Metabolomics offers an innovative strategy to identify new diagnostic, predictive and prognostic biomarkers, as well as treatment targets in sarcoma.

Figure 1.

The workflow of metabolomics in sarcoma. There are several general steps in the metabolomics studies, including: sample collection, sample preparation and metabolite extraction, and data acquisition, analysis and interpretation.

Table 1
Comparisons of three major approaches in metabolomics

Approaches	Advantages	Disadvantages	Functions	Refs.
Metabolite/metabolic profiling	<ul style="list-style-type: none"> ❖ Most commonly used ❖ Not targeted ❖ Identify unknown metabolites 	<ul style="list-style-type: none"> ✓ Semi-quantitative ✓ Majority of peaks are not identifiable ✓ Difficult informatics ✓ Medium throughput 	<ul style="list-style-type: none"> ➤ Establish the metabolome of cell ➤ Investigation of selected biochemical pathways in a cell line or tissue 	[24-26,23]
Metabolic fingerprinting	<ul style="list-style-type: none"> ❖ Not targeted ❖ Qualitative and quantitative ❖ Directly applicable to pattern recognition ❖ Highest throughput 	Can't identify Unknown metabolites	<ul style="list-style-type: none"> ➤ A rapid classification of the metabolism in a cell line or tissue according to their origin or their biological relevance ➤ Discover specific metabolic patterns of diseases ➤ Discover diagnostic biomarker 	[24-26,23]
Targeted analysis	<ul style="list-style-type: none"> ❖ Quantitative ❖ Identified metabolites ❖ Extreme sensitivity ❖ Low limit of detection ❖ High throughput 	<ul style="list-style-type: none"> ✓ Limited number of targeted compounds ✓ Targeted compounds must be available as purified for calibration or Targeted compounds must be purified for calibration 	<ul style="list-style-type: none"> ➤ Study the effect of any alteration directly ➤ Analyze the substrate and/or the direct product of the corresponding encoded enzyme 	[24-26,23]

Abbreviations: Refs references

Table 2
Comparisons of the major analytical methods in metabolomics

Analytical methods	Advantages	Disadvantages	Refs.
LC-MS	❖ High sensitivity		
	❖ No derivatization required	✓ Slow	
	❖ Many modes of separation available	✓ Limited commercial libraries	[30,27]
	❖ Large sample capacity		
GC-MS	❖ High sensitivity	✓ Slow	
	❖ Robust	✓ Often requires derivatization	
	❖ Large linear range	✓ Many analytes thermally-unstable or too large for analysis	[30,27]
	❖ Large commercial and public libraries		
CE-MS	❖ High separation power		
	❖ Small sample requirements		
	❖ Rapid analysis	✓ Limited commercial libraries	
	❖ Can analyse neutrals, anions and cations in single run	✓ Poor retention time reproducibility	[30,27]
	❖ No derivatization		
NMR spectroscopy	❖ Fast analysis	✓ Low sensitivity	
	❖ High resolution	✓ Convoluted spectra	
	❖ No derivatization needed	✓ More than one peak per component	[30,27]
	❖ Non-destructive	✓ Libraries of limited use due to complex matrix	
	❖ Less expensive		
	❖ Requires minimal handling		

Abbreviations: LC-MS liquid chromatography coupled with mass spectrometry; GC-MS gas chromatography coupled with mass spectrometry; CE-MS capillary electrophoresis coupled with mass spectrometry NMR nuclear magnetic resonance; Refs references

Table 3

Metabolomics studies in osteosarcoma (OS)

Samples	Analytical technologies	Database	Data analysis methods	Main results	Applications	Ref.
OS serum and urine (24), benign bone tumor serum and urine (19), healthy serum and urine (32)	GC-TOF-MS (serum); GC-MS (urine)	NIST library	Peak detection and alignment, PCA, PLS, OPLS-DA	<ul style="list-style-type: none"> The metabolism TCA cycle, glycolysis and lipid were downregulated. The metabolism of amino acid metabolism, glutathione metabolism and polyamine were upregulated. 	<ul style="list-style-type: none"> Diagnostic biomarker Treatment target 	[44]
TAp73 α -induced and noninduced human SAOS-2 OS cell	TOF-MS	KEGG pathway database	Peak detection and alignment, targeted MRM analysis	The metabolisms of glycolysis, Krebs cycle, glutathione homeostasis, pentose phosphate pathway, purine, arginine-citrulline-NO were upregulated.	<ul style="list-style-type: none"> Simultaneous application of proteomics, lipidomics Diagnostic biomarker Treatment target 	[45]
TAp73 OS SaOS-2 Tet-On cell lines, control cells treated with vehicle	GC-MS; LS-MS	All the upregulated metabolites in our analysis, KEGG and Reactome database	Peak detection and alignment	The metabolism of glycolysis, amino acids, acetyl-CoA, polyamine, and membrane phospholipids were upregulated.	Treatment target	[46]
OS serum (24), healthy control serum (32)	GC-TOF-MS	KEGG pathway database	Peak detection and alignment, OPLS-DA	Glycolysis metabolism was enriched.	<ul style="list-style-type: none"> Integrated with transcriptomics analysis Diagnostic biomarker 	[22]
CDDP-treated human OS MG-63 cells	¹ H HR-MAS-NMR spectroscopy	Bruker Bioreference spectral database	PCA, PLS-DA, SRV analysis	<ul style="list-style-type: none"> The metabolism of lipids and choline were upregulated. The metabolism of glutamate, taurine, and polyalcohols were downregulated. 	<ul style="list-style-type: none"> Predictive biomarkers of chemoresistance Treatment target 	[34]
DOX-treated, MTX-treated, CDDP-treated, and control MG-63 cell	¹ H HR-MAS-NMR spectroscopy	Bruker Bioreference database & the human metabolome database	Peak detection and alignment, PCA, PLS-DA, t-test	<ul style="list-style-type: none"> CDDP upregulated lipids metabolism. MTX and DOX downregulated choline metabolism. 	<ul style="list-style-type: none"> Predictive biomarkers of chemoresistance Treatment target 	[35]

Samples	Analytical technologies	Database	Data analysis methods	Main results	Applications	Ref.
				❖ DOX and MTX upregulated amino acids metabolism.		
Human OS MG-63 cell	GC-TOF-MS, GC-MS	-	Peak alignment, normalization, PCA, PLS-DA, OPLS	The metabolism of serine/glycine was upregulated.	❖ Predictive biomarkers of chemoresistance ❖ Treatment target	[47]
Mouse serum from primary tumor (32), metastatic tumor (32) and controls (23)	GC-TOF-MS	NIST, Wiley, NBS database	Peak detection and alignment, PCA, PLS-DA	The metabolism of amino acids, TCA cycle, free fatty acids, sugars and polyols were upregulated during lung metastasis.	Diagnostic biomarker for primary OS and lung metastasis	[48]

Abbreviations: LC liquid chromatography, MS mass spectrometry, GC gas chromatography, TOF-MS time-of-flight mass spectrometry, PCA principal component analysis, PLS partial least-squares, OPLS orthogonal partial least-squares, DA discriminant analyses, TCA tricarboxylic acid, TA transactivation-proficient, HR-MAS high resolution magic angle spinning, NMR nuclear magnetic resonance, PQN probabilistic quotient normalization, DOX doxorubicin, MTX methotrexate, CDDP cisplatin, MRS magnetic resonance spectroscopy, LC/MS/MS liquid chromatographytandem mass spectrometry, SAM significance analysis of microarrays, SRV Statistical recoupling of variables, MRM multiple reaction monitoring, CoA coenzyme A

Table 4**Metabolomics studies in other types of sarcomas**

Sarcoma	Samples	Analytical technologies	Database	Data analysis methods	Main results	Applications	Ref.
LS	Human LS cell	LC-MS	-	PCA, supervised hierarchical clustering	The metabolism of nucleosides, amino acids and lipids were downregulated.	<ul style="list-style-type: none"> ❖ Diagnostic biomarker ❖ Treatment target 	[52]
Soft tissue sarcoma (LES, SS, LS)	Formalin-fixed, paraffin-embedded tumor tissue	LC-MS	Metabolic pathway associated metabolite sets	Peak detection and alignment, PCA, unsupervised hierarchical clustering, pathway analysis	The metabolism of glycolysis, glutamate metabolism, and the citric acid cycle were upregulated.	Diagnostic biomarkers	[28]
LES	Human cell lines SKLMS1 and SKUT1	CE-MS	-	-	Arginine starvation of ASS1-deficient tumors causes an increase in serine biosynthesis, glutamine anaplerosis, and oxidative phosphorylation with a simultaneous decrease in aerobic glycolysis	Treatment target	[53]
High grade sarcomas (OS, LES, MFS, UPS)	33 formalin-fixed, paraffin-embedded tumor tissue	MSI	Human metabolome database	-	Inositol cyclic phosphate, carnitine	<ul style="list-style-type: none"> ❖ Overall survival prognostic biomarker ❖ Metastatic prognostic biomarker 	[54]

LS liposarcoma, LES leiomyosarcoma, SS synovial sarcoma, LC liquid chromatography, MS mass spectrometry, PCA principal component analysis, CE capillary electrophoresis, MFS myxofibrosarcoma, UPS undifferentiated pleomorphic sarcoma, MSI Mass Spectrometry Imaging