Osteosarcoma Biomarkers Discovery Using "Omics" Approaches

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Abstract

Osteosarcoma is the most common malignant primary cancer of bone tissue affecting mostly children and young adults. Nowadays, reliable circulating or cellular/tissue biomarkers do not exist for early diagnosis, drug resistance, and relapses of osteosarcoma. Post-genomics represents an invaluable tool to disclose cancer complexity at a molecular as well as to discover novel diagnostic and prognostic biomarkers.

Although "omics" research on osteosarcoma has only been undertaken recently in respect to that on many other tumor types, these studies have brought

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to light several potential molecular biomarkers that represent the basis to develop novel and better strategies for early detection, outcome prediction, detection of disease recurrence, and therapeutic approach.

In this chapter, the discovery of such molecular markers through the emerging omics technologies, including miRNA-omics, transcriptomics, and proteomics, will be extensively reviewed.

Keywords

Osteosarcoma • Post-genomics • Omics approaches • miRNA • Transcriptomics • Proteomics • Biomarker

List of Abbreviations

1DE	Monodimensional polyacrylamide gel electrophoresis
2D-DIGE	Two-dimensional difference in gel electrophoresis
2DE	Two-dimensional polyacrylamide gel electrophoresis
CSC	Cancer stem cell
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FACS	Fluorescence-activated cell sorting
FT-ICR	Fourier transform ion cyclotron resonance
IHC	Immunohistochemistry
iTRAQ	Isobaric tags for relative and absolute quantitation
LC	Liquid chromatography
LTQ	Linear ion trap
miRNA	Micro RNA
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
OB	Osteoblast
OC	Osteochondroma
OS	Osteosarcoma
PCA	Principal component analysis
PMF	Peptide mass spectrometry
q RT-PCR	Quantitative real time PCR
QToF	Quadrupole time of flight
SELDI-ToF/MS	Surface-enhanced laser desorption/ionization time of flight mass spectrometry
WB	Western blotting

Key Facts of Osteosarcoma

- Osteosarcoma is the most common type of bone cancer, which develops in growing bones and occurs more frequently in children and adolescents.

- The most common early signs of osteosarcoma are pain and swelling. The presence of a bone tumor has to be confirmed by a complete medical examination including blood test, since bone tumors can be associated with increased levels of certain enzymes in the blood; X-rays and other scans of the bone(s); and then a biopsy (removal of a sample of tissue) that will be examined by a pathologist to determine whether it is cancerous and if so what type of cancer it is.
- Osteosarcomas can be localized or metastasize to other parts of the body (mainly lungs). Microscopic spreads can occur even at the early phases of cancer progression, when the primary tumor has a very small size.
- Modern treatments of osteosarcoma require surgery (to remove all visible tumor tissue) and chemotherapy, given before (to shrink tumor size and to prevent metastasis) and after surgery (to kill cancer cells not completely removed by surgery). Many factors including site and location of the main tumor and other individual factors affect the type of surgery. When it is possible, limb-sparing procedures by an artificial device (endoprosthesis) or bones from other places in the body (bone graft) are preferred to amputation.
- Several factors affect the prognosis of osteosarcoma patients, including cancer spreading, size and location of tumor, type of osteosarcoma, surgery outcome, responsiveness to chemotherapy, and patient's general health.

Definitions of Words and Terms

Biological marker (Biomarker)	A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. (Biomarker Definitions Working Group – 1998).
miRNA	miRNAs are short noncoding RNA molecules that regulate gene expression at the posttranscriptional level by binding to the 3′ untranslated regions of target messenger RNAs. To date, nearly 2000 human miRNAs have been identified and any single miRNA can regulate dozens or hundreds of target genes.
Proteome	Large-scale inventory of the proteins expressed in cells, tissues, or organisms. Proteome reflects a specific developmental stage or physiological condition.
Proteomics	Comprehensive study of a specific proteome with the aim to catalog all protein species, to determine their structure and function, and to quantify the changing expression levels of each protein species during development and under different

	conditions. Proteomics approaches are conducted by means of high-throughput technologies.
Transcriptome	Large-scale inventory of the RNA transcripts (mRNAs, noncoding RNAs, and small RNAs) produced by the genome in cells, tissues, or organisms. Transcriptome reflects a specific developmental stage or physiological condition.
Transcriptomics	The study of a specific transcriptome with the aim to catalog all species of transcript, to determine the transcriptional structure of genes, and to quantify the changing expression levels of each transcript during development and under different conditions. Transcriptomics approaches are conducted by means of high-throughput technologies.

Introduction

Osteosarcoma (OS) is a rare neoplasm of bone that affects mainly young patients. Since OS have a high tendency to metastasize, they are classified among the most frequent sources of cancer-related death in childhood tumors (Botter et al. 2014). Despite the survival rate of OS patients has been improved as a result of refined surgical techniques and multiagent chemotherapy, the survival of patients that develop metastases still remains low (Anninga et al. 2011). Therefore, a more detailed understanding of the molecular mechanisms and specific identifying of biomarkers involved in tumor initiation, progression, and metastasis formation is of immediate importance to develop new and improved treatment strategies for OS.

Currently the diagnosis of OS occurs around 4 months from the onset of symptoms. Diagnosis of OS is based, after a first complete medical history of the patient, on imaging analysis, including radiographs, magnetic resonance imaging, bone scintigraphy, and biopsy which provide a definite diagnosis and grading/staging of the tumor. So far, reliable OS circulating markers do not exist. In fact, alkaline phosphatase (ALP) exhibits high plasmatic level only in 40% of cases, while lactate dehydrogenase (LDH) is elevated in around 30% of cases. These laboratory values also possess a moderate prognostic relevance: normal ALP and LDH levels in chemonaive patients have been associated with 5-year disease-free survival and a longer time to disease recurrence (Geller and Gorlick 2010). However, the most important prognostic factors in OS are represented by the presence of metastatic disease at the time of diagnosis and the histological response to preoperative chemotherapy.

Nowadays, the greatest challenge in OS management is the lack of reliable markers able to detect the tumor at an early stage, when there is a better chance for its treatment, or to predict the prognosis or the response to chemotherapy.

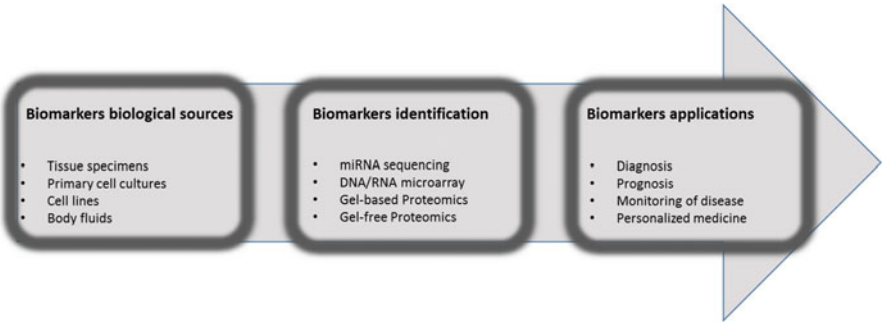

Fig. 1 Targeted "OMICS" approaches to biomarkers discovery. Different biological samples are collected from patients with OS and represent the source of molecular biomarker. Global profiles are obtained using high-throughput post-genomics technologies and then analyzed to identify candidate biomarkers

In the last few years, the significant progress in "omics" technologies (epigenomics, transcriptomics, and proteomics), allowing the simultaneous detection of thousands of molecular species in a large amount of biological samples, provided researchers with the opportunity to discover a variety of biomarkers with diagnostic and prognostic purposes. In this regard, the development of bioinformatics analytical tools suitable to mine the massive flood of data provided by high-throughput experiments is mandatory to integrate different "omics" approaches as well as to achieve robust and reliable finding with clinical relevance as well as to get novel clues for understanding cancer biology and pathophysiology (Bernardini et al. 2012, 2014).

Moreover, the evaluation of tumor-specific "omics" profiles may also allow the development of more efficient tools for cancer therapy through the identification of novel molecular targets and thus the development of personalized therapies (Fig. 1).

The main aim of the present chapter is to systematically summarize the most relevant post-genomic studies related to post-genomic biomarkers discovery in OS.

MicroRNAs

MicroRNAs (miRNAs) are a novel class of biomarkers, which could be helpful for OS diagnosis and determination of optimal treatment.

miRNAs are short noncoding RNA molecules ~22 nucleotides long that are synthesized by RNA polymerase II or III from endogenous transcription units. They regulate gene expression at the posttranscriptional level by binding to the 3′ untranslated regions (3′ UTRs) of target messenger RNAs (Ambros 2004). To date, nearly 2000 human miRNAs have been identified (miRBase, Homo sapiens miRNAs database, Manchester University), and any single miRNA can regulate dozens or hundreds of target genes (Rana 2007).

In the context of cancer cells, miRNAs can act as oncogenes (oncomiR) or tumor suppressor genes (anti-oncomiR) based on their inhibition of tumor-suppressive and oncogenic mRNAs, respectively, and expression deregulation of one or more miRNAs was demonstrated to be involved in development and progression of cancer (Calin et al. 2002; Sotiropoulou et al. 2009). The expression profiling of miRNAs is already used into cancer clinics as diagnostic and prognostic biomarkers to assess tumor initiation, progression, and response to treatment (Reddy 2015).

miRNAs Expression in OS

Since the recent discovery of the class of miRNAs in humans (Lagos-Quintana et al. 2001), the interest in the field has grown rapidly, and during the last 5 years the number of papers devoted to miRNAs in OS increased exponentially (Fig. 2).

In a recent study, comparison of 80 pairs of OS and corresponding noncancerous bone tissues revealed that miR-34a and miR-192 were downregulated in tumors, and OS patients with low miR-34a and miR-192 expression had shorter disease-free survival (Wang et al. 2015b). Thus, miR-34a and miR-192 are potential biomarkers associated with unfavorable prognosis. Interestingly, the expression of miR-34a is highly induced by p53 following DNA damage and oncogenic stress, and reduction of miR-34 function attenuates p53-mediated cell death (He et al. 2007). Moreover, in OS models, miR-34a inhibits proliferation, angiogenesis, and metastasis of tumor cells by targeting Notch-1, mTOR, c-Met, MDM4, and Eag1 (Li et al. 2013; Tian et al. 2014b; Wu et al. 2013; Yan et al. 2012).

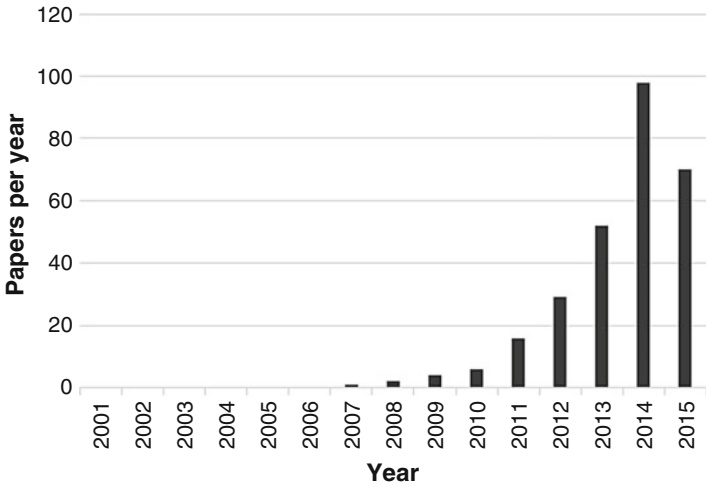

Fig. 2 Number of scientific publications related to miRNA investigation in OS. Annual number of peer-reviewed papers published with "osteosarcoma" and "miRNA" in their titles, keywords, or abstracts from 2001 (Year of discovery of the miRNA class in humans (Lagos-Quintana et al. 2001)) to May 2015 (Pubmed database)

In a sample of 52 patients, miRNA-22 was identified as a novel potential biomarker of unfavorable prognosis in OS (Wang et al. 2015a). In fact, miR-22 is downregulated in OS in comparison with noncancerous bone tissues, and its low expression level correlates with recurrence, metastasis, chemotherapy response, and poorer overall survival and DFS. miR-22 seems to act as tumor suppressor gene by targeting the 3′UTR of high-mobility group box 1 (HMGB1) and inhibiting its translation. In OS cells, high levels of HMGB1 (due to miR-22 downregulation) promote autophagy and consequent drug resistance (Guo et al. 2014).

As example of upregulated miRNAs in OS, miR-27a was found to be prognostic of metastatic disease in a sample of 18 patients (Jones et al. 2012). miR-27a is described to promote metastasis in OS, at least in part, through targeting the tumor suppressor CBFA2T3, which is downregulated in a majority of patients (Salah et al. 2015).

Up to now, several other miRNAs have been found to be implicated in OS (Zhang et al. 2015), and a tool was needed to manage the information regarding the expression patterns. To this aim, Korsching and coworkers constructed the Osteosarcoma Database, which provides a structured, annotated, and easy accessible overview of the protein-coding and miRNAs genes whose expression correlates with disease progression and that might be used as biomarkers (http://osteosarcoma-db.uni-muenster.de). At the time of the last update (October 2013), the Osteosarcoma Database contains 911 protein-coding genes and 81 microRNAs associated with OS according to 1,331 PubMed abstracts. The Osteosarcoma Database offers "the possibility to rank and sort the literature according to various parameters, including therapeutic and prognostic value of specific genes and microRNA and the type of sample used" (Poos et al. 2014).

miRNAs Detection Methods

Quantitative real-time PCR (qRT-PCR) technique is the most popular reference test to quantify miRNA expression, because of its speed, simplicity, low cost of exercise, and high sensitivity and specificity. The disadvantage is that this technique is time consuming and laborious if large number of miRNA has to be analyzed. Microarrays or microRNA sequencing (miRNA-seq), instead, are used when high throughput is desired, even if they need more complex steps of standardization and validation. Microarray platforms allow the analysis of thousands of miRNAs in a single experiment, and it is widely used in order to detect and quantify miRNAs. miRNA-seq uses next-generation sequencing technology to massively sequence miRNAs; it is relatively recent but is replacing microarrays. This miRNA-seq technology has the advantage of quantifying and identifying known miRNAs, as well as novel miRNAs.

A major difficulty in miRNA quantification from patient tissues is the availability of frozen samples. Recently, Spentzos and colleagues overcome this problem and published a large OS profiling study (Kelly et al. 2013). They used the Illumina cDNA-mediated annealing, selection, extension, and ligation (DASL) assay to analyze the expression of 1,146 miRNAs from the partially degraded RNAs

extracted from 91 formalin-fixed, paraffin-embedded (FFPE) OS diagnostic biopsy specimens and identified a cluster of miRNAs with predictive value for OS recurrence and survival. This cluster is located at the 14q32 locus, already linked to this type of cancer. Through this technology, they also identify nonoverlapping miRNA profiles predictive of chemoresponse.

Circulating miRNAs

miRNAs are not only regulators of gene expression in the same cell in which they are synthesized, but they can be secreted and transferred horizontally between cells, assuming also a role in intercellular communication and long-distance signaling, regulating target RNAs in recipient cells (Chen et al. 2012). Circulating miRNAs have been found in serum, plasma, and other body fluids and represent attractive biomarkers in noninvasive serological tests for the diagnosis or prognosis of cancer. This type of analysis presents the advantage of easier samples achievement and, consequently, it allows analyzing larger cohort of patients. Recent findings on circulating miRNA associated with OS are summarized in Table 1.

In some interesting example, such as miR-9 and miR-214, the differential expression of miRNAs and their prognostic value in OS were described for both plasma and tumoral tissue (Allen-Rhoades et al. 2015; Fei et al. 2014; Wang et al. 2014; Xu et al. 2014).

In conclusion, it is clear that the expression profiles of circulating miRNA are useful as biomarkers for OS diagnosis, prognosis, and chemoresponse. But despite the large progress in the field, a lot of work is still needed to identify, characterize, and validate the most predictive biomarkers, and several research findings have to be clarified, such as the opposite expression profile found in different studies for miR-199a-3p in plasma of patients (Lian et al. 2015; Ouyang et al. 2012).

Transcriptomics

Over the last few years, the analysis of differentially expressed genes by microarray combined with bioinformatics analysis has been used to identify key genes and cellular signaling pathways involved in OS progression and metastasis. However, OS genome-wide studies resulted extremely hard due to the rarity of the disease, the high genomically unstable OS cells, and the heterogeneity of tumor clinical samples (Kuijjer et al. 2013). In a recent review, the challenges of high-grade OS data analysis have been discussed (Kuijjer et al. 2013), giving an overview of the major findings on DNA/RNA microarray reports on OS. Therefore, here we will only review the most recent findings on OS obtained by microarray analyses.

Genetic regulation is pivotal for the occurrence and progression of tumors and the development of advanced technologies, such as serial analysis of gene expression

Table 1 Circulating miRNAs associated with OS

Circulating miRNAs	No. of patients	Expression	Correlation with	References
miR-9	118	Upregulated	Tumor size, metastasis, overall survival	Fei et al. 2014
miR-21	80 + 65	Upregulated	Metastasis, tumor subtype, Enneking stage, chemoresistance	Ouyang et al. 2012 Yuan et al. 2012
miR-34b	133	Downregulated	Metastasis	Tian et al. 2014a
miR-133b	100	Downregulated	Tumor grade, metastasis, recurrence, survival	Zhang et al. 2014b
miR-143	80	Downregulated	Metastasis, tumor subtype	Ouyang et al. 2012
miR-148a	89	Upregulated	Tumor size, metastasis, survival	Ma et al. 2014
miR-195-5p	90	Upregulated	Metastasis	Lian et al. 2015
miR-196a	100	Upregulated	Tumor grade, metastasis, and recurrence	Zhang et al. 2014a
miR-196b	100	Upregulated	Tumor grade, metastasis, and recurrence	Zhang et al. 2014a
miR-199a-3p	90	Upregulated	Metastasis, chondroblastic subtype	Lian et al. 2015
miR-199a-3p	80	Downregulated	Metastasis	Ouyang et al. 2012
miR-205-5p	40	Downregulated		Allen-Rhoades et al. 2015
miR-206	100	Downregulated	Tumor grade, metastasis, recurrence, survival	Zhang et al. 2014b
miR-214	40	Upregulated	Survival	Allen-Rhoades et al. 2015
miR-320a	90	Upregulated	Osteoblastic subtype	Lian et al. 2015
miR-335-5p	40	Upregulated		Allen-Rhoades et al. 2015
miR-374a-5p	90	Upregulated		Lian et al. 2015
miR-574-3p	40	Upregulated		Allen-Rhoades et al. 2015

provided the means to identify putative biomarkers for a large number of tumors, including OS. A summary of the most important genes and signaling pathways involved in the formation of OS and identified by genome-wide studies are provided

in Tables 2 and 3. By high-density oligonucleotide microarray, potential biomarkers of both prognostic and therapeutic significances were identified in OS cell lines (Zou et al. 2012). Interestingly, among them cancer testis antigens, such as melanoma antigen family A (MAGEA), were significantly increased and associated with a high risk of metastasis and poor survival. A meta-analysis study on different gene expression data of OS has allowed to detect differences between control tissues and OS, such as enrichment in focal adhesion pathway (Yang et al. 2014).

Recently, several microarray and meta-analysis studies have been carried out to unveil potential biomarkers for metastatic OS. In a screening using a DNA microarray, differentially expressed genes were identified and classified as upregulated, most significantly in cytoskeleton organization, and downregulated, mainly in wound healing (Diao et al. 2014). Seventeen differentially expressed genes were described to be metastasis related and considered as important players in tumor progression of osteoblastic OS, the predominant phenotype of the disease (Muff et al. 2012). New putative targets were supposed to be useful for the diagnosis and

Table 2 Major genes identified in genome-wide and microarray studies in OS

Gene	Function	References
IGFBP5	Tumor suppressor	Su et al. 2011
WIF1	Tumor suppressor	Kansara et al. 2009
LSAMP	Tumor suppressor	Kresse et al. 2009, Yen et al. 2009
Cyclin E3	Oncogene	Lockwood et al. 2011
RUNX2	Oncogene	Kresse et al. 2012, Sadikovic et al. 2009
DOCK5	Tmor suppressor	Sadikovic et al. 2009
TNFRSF10A	Tumor suppressor	Sadikovic et al. 2009
DLX5	Oncogene	Kresse et al. 2012
CXCL5	Tumor suppressor	Kresse et al. 2012
PRAME	Oncogene	Kresse et al. 2012, Zou et al. 2012
NKD2	Tumor suppressor	Zhao et al. 2015

IGFBP5 insulin-like growth factor binding protein 5, *WIF1* Wnt inhibitory factor 1, *LSAMP* limbic system-associated membrane protein, *VEGF* vascular endothelial growth factor, *RUNX2* runt-related transcription factor 2, *DOCK5* dedicator of cytokinesis 5, *TNFRSF10A* tumor necrosis factor receptor superfamily, member 10, *DLX5* distal-less homeobox 5, *CXCl5* chemokine (C-X-C motif) ligand 5, *PRAME* preferentially expressed antigen in melanoma, *NKD2* naked cuticle homolog 2

Table 3 Major signaling pathways identified in genome-wide and microarray studies in OS

Signaling pathway	References
Macrophage-associated genes correlated with better metastasis-free survival	Buddingh et al. 2011
DNA replication network	Cleton-Jansen et al. 2009, Sadikovic et al. 2009
Amplification of the VEGF pathway genes	Yang et al. 2011
Deregulation of the cell cycle	Kuijjer et al. 2012
Apoptosis, signal transduction	Kuijjer et al. 2012

treatment of metastatic OS, such as alpha-2-macroglobulin (A2M) and its interactive proteins (Niu et al. 2014), metalloproteinase 1 (MMP1), smoothened (SMO), Ewing sarcoma breakpoint region 1 (EWSR1), fasciculation and elongation protein 1 (FEZ1), brain-selective kinase 2 (BRSK2), aldo-keto reductase family 1 member B10 (AKR1B10) (Yao et al. 2015), brain-specific angiogenesis inhibitor 2 (BAI2), formin-like 1 (FMNL1), dual-specificity phosphatase 7 (DUSP7), transient receptor potential melastatin 2 (TRPM2) (Wang 2015), epiregulin (EREG), and carbohydrate (N-acetylglucosamine-6-O) sulfotransferase 2 (CHST2) (Chen et al. 2011). Moreover, by microarray analysis performed on a mouse model of localized and metastatic OS, it was demonstrated that downregulation of naked cuticle homolog 2 (NKD2) expression plays an important role in driving OS tumor growth and metastasis (Zhao et al. 2015).

Microarray analysis was also utilized to gain insights into the molecular mechanisms underlying signaling pathway or set of proteins known to be involved in OS progression and metastasis. Ribosomal protein S3 (RPS3) was identified as a downstream factor of GLI2 that mediates migration and invasion of OS (Nagao-Kitamoto et al. 2015); ΔNp63α, the predominant p63 isoform expressed in invasive OS cells, turned out to be necessary for the regulation of GLI2 expression to promote its oncogenic properties (Ram Kumar et al. 2014); and special AT-rich binding protein 2 (SATB2), highly expressed in OS, revealed the ability to regulate epithelial protein lost in neoplasma (EPLIN) and genes involved in cytoskeleton dynamics to increase OS migration and invasion (Seong et al. 2014).

OS is considered to be a differentiation disorder of mesenchymal stem cells, which produce defective, immature bone. Despite this simple definition, OS is highly heterogeneous and is subdivided into numerous different histological subtypes. Currently these subtypes are classified on the basis of morphological and histological criteria, but the identification of biomarkers that characterize each subtype would be of major importance to improve therapeutic and prognostic outcomes. Using microarray-based differential expression and gene set analysis, a different gene expression pattern was identified between osteoblastic and nonosteoblastic OS subgroups (Kubista et al. 2011), while gene expression analysis allowed to identify genes involved in plasticity of anoikis-resistant OS subgroups characterized by a rapid development of chemoresistance and altered growth rate, mimicking the early stages of latent metastasis (Foley et al. 2015).

OS is the most common primary bone malignancy in dogs. Canine OS shares several traits with human OS, making dogs a valuable comparative model that has strong potential applicability to the human disease (Sutter and Ostrander 2004). Indeed, using gene expression microarray analysis on canine OS samples, potential new biomarkers and novel pathways that may be targeted for therapeutic intervention were identified (O'Donoghue et al. 2010).

Gene expression profiling by microarray combined with other techniques resulted successful in several studies. Indeed, expression microarray analysis combined with the investigation of focal copy number aberrations has allowed identifying CKLF-like Marvel transmembrane domain containing 8 (CMTM8) as a new candidate tumor suppressor and G protein-coupled receptor 177 (GPR177) as a new putative

oncogene in OS (Both et al. 2014). Furthermore, combining proteomic analysis with previously obtained cDNA microarray results allowed detecting aldolase A fructose-bisphosphate (ALDOA) and sulfotransferase family cytosolic 1A phenol-preferring 3 (SULT1A3) as predictors of clinical outcomes for OS patients (Chen et al. 2014), while microarray-based comparative genomic hybridization (aCGH) allowed to gain a comprehensive understanding of the key driving pathways for OS, elucidating the contradictory role of Wnt signaling (Du et al. 2014), and identifying a functional crosstalk between vascular endothelial growth factor (VEGF) and runt-related transcription factor 2 (RUNX2) essential for the pathogenesis and angiogenesis of the disease (Yang et al. 2013).

Finally, although in some studies microarray failed to predict biomarkers for OS patients' outcome (Sabile et al. 2013), for researchers it certainly provides an useful tool to characterize the altered expression of genes involved in the development and behavior of OS subtypes and to identify the gene signature of an individual OS patient revealing distinct signaling events, which might account for the biological features specific for each tumor type.

Proteomics

Proteomic approaches to cancer research offer several advantages in respect to other high-throughput technologies such as genomics or transcriptomics. In addition to global protein profiling and protein identification, proteomics provides powerful tools to investigate the complexity of these highly dynamic macromolecules. In fact, disease-associated phenotypic alterations are consequences not only of deregulated (increasing/decreasing) expression of proteins but also of functional regulations by various processes such as proteins degradations, posttranslational modifications (e.g., phosphorylation, glycosylation, methylation), involvement in complex structures, and differential compartmentalization (e.g., nuclear localization).

Moreover, proteomic approaches can be applied to a variety of biospecimens ranging from biological models, such as cell lines, primary cell cultures, or animal models of disease, to clinical samples, including serum/plasma, urine, spinal fluid, synovial fluid, and tissue.

Comprehensive analysis of proteomic data from cancer patients' samples has notably improved our understanding of tumor pathogenesis and treatment, uncovering the different processes involved in cancer development and progression, along with the identification of novel target for cancer therapy.

The discovery of biomarkers with clinical relevance using proteomics is affected by several critical challenges, in particular the biological variability among patients' samples and the huge dynamic range of biomarkers concentration in biological fluids. In addition to these, another major obstacle to be taken into account is the thousands of cancer-associated proteins detected by high-throughput proteomic approaches that have to be properly validated.

Nevertheless, in the last decade proteomic approaches lead to the discovery of clinically relevant biomarkers for several types of cancers such as breast, esophageal, lung, liver, and colorectal cancer. All these biomarkers possess high values of specificity and sensitivity and represent unvaluable tool to be used for screening, early detection, and prediction of response to therapy in oncology (Sallam 2014).

Proteomics technologies include gel-based methods (1DE, 2DE, and 2D-DIGE), gel-free methods based on mass spectrometry (SELDI and MALDI ToF/MS, LC-MS/MS), or based on array (antibody array, reverse phase protein microarray (RPMA)) and bioinformatics.

Several proteomic approaches have been applied to OS research to elucidate the molecular mechanism underlining the development and progression of the diseases and also to identify new molecular markers for early diagnosis, prognosis, and chemotherapy responsiveness (Table 4).

To address these aims, different types of human biological samples have been used such as OS cell lines and primary cell cultures, OS bone tissue, or serum (Table 4).

Biomarkers from OS Cells

Comparative 2-DE was applied to total protein extract of OS cell lines (i.e., SaOS-2, U2OS, and IOR/OS9) and primary or SV-40 immortalized osteoblastic cells (i.e., hFOB1.19) (Spreafico et al. 2006; Guo et al. 2007; Liu et al. 2009). All three studies report a list of proteins whose expression was found altered in OS cell lines in respect to healthy counterparts. However, when comparing the results, a total absence of overlapping is noticeable. This inconsistency could rely on the type of control samples used by the authors, immortalized cells (Guo et al. 2007) or primary cells extracted from different anatomical sites (Spreafico et al. 2006; Liu et al. 2009), or on slight differences in the experimental procedures.

Subproteomic analyses of OS cell lines were also performed with particular attention to membrane (Zhang et al. 2010; Hua et al. 2011) and surface exposed proteins (Posthumadeboer et al. 2013). The group led by Cai applied a double approach to identify plasma proteins able to differentiate MG63 OS cells from hFOB1.19 cells (Zhang et al. 2010; Hua et al. 2011): CD151 was selected by a quantitative gel-free analysis (iTRAQ-LC/MS/MS) combined to bioinformatics (Zhang et al. 2010), while NDRG1 was identified by a gel-based approach (Hua et al. 2011). Both marker candidates were then validated by WB and IHC. Posthumadeboer et al. identified EPHA2 receptor as the most abundant surface proteins in several OS cell lines (SaOS-2, MG63, U2OS, and SaOS-2 LM7) and significantly overexpressed in OS cells and tissues in respect to normal samples (Posthumadeboer et al. 2013).

OS-specific proteins were also investigated by Folio in primary cells isolated from five paired samples of OS tumor and normal bone tissue (Folio et al. 2009). 2DE global protein profiling showed the upregulation of 56 protein spots in

Table 4 Protein biomarkers identified by proteomics in OS

Proteins	Application	Biological samples	Validation	Technique	References
Pyruvate kinase M1, L-lactate dehydrogenase B chain, triose phosphate isomerase 1, creatine kinase B chain, heat shock protein 90, 150 kDa oxygen-regulated protein, retinoblastoma-binding protein 4, alkaline phosphatase	Diagnosis	OS cell line (SaOS-2) and primary OB cells	No	2DE and PMF	Spreafico et al. 2006
Activator of 90 kDa heat shock protein, ATPase homolog 1 AHA1, and stomatin-like protein 2	Diagnosis	OS cell lines (SAOS-2, U2-OS and IOR/OS9) and OB cells (SV-immortalized hFOB1.19)	No	2DE and PMF	Guo et al. 2007
Cytochrome b-c1 complex subunit 1, ubiquitin carboxyl-terminal hydrolase isozyme L1, peroxiredoxin-4	Diagnosis	OS cell line (SaOS-2) and primary OB cells	Yes, WB	2DE and PMF	Liu et al. 2009
Ezrin, crystallin b chain	Diagnosis	Chemonaive primary OS and paired normal OB cells	Yes, RT-PCR and WB	2DE and nano-LC-ESI-QToF MS/MS	Folio et al. 2009
CD 151 (Membrane glycoprotein SFA-1)	Diagnosis	OS MG63 cells and OB cells (SV-immortalized hFOB1.19): plasma membrane proteins	Yes, IHC	iTRAQ-LC-MS/MS	Zhang et al. 2010
NDRG1	Diagnosis	OS MG63 cells and OB cells (SV-immortalized hFOB1.19): plasma membrane proteins	Yes, WB IHC	2DE and nano-LC-MS/MS	Hua et al. 2011
Ephrin type-A receptor 2 (EPHA2)	Diagnosis	OS cell lines (MG 63, U2OS, Cal-72, SaOS-2, SaOS-LM7) and primary OB cells (ORT-1, Hum31, Hum54, Hum63, Hum65: surface proteins)	Yes, FACS and IHC on tissue microarray	1DE and nano-LC-LTQ-FT MS/MS	Posthumadeboer et al. 2013

Aldolase A and sulfotransferase 1A3/1A4	Prognosis	OS cell lines with different metastatic potential (F5M2 and F4)	Yes, WB and ICH	2D-DIGE and PMF	Chen et al. 2014
Translationally controlled tumor protein, malate dehydrogenase, CBX3, dihydropyrimidinase-related protein 2, fructose-bisphosphate aldolase C	Diagnosis	OS cancer stem cells CHA59	CBX3: Yes, RT-PCR	2DE and LC-MS/MS	Saini et al. 2012
Activation of MAPKs pathway	Diagnosis	OS CSC (3-AB OS) and its parental OS cell line (MG-63)	No	Antibody array and knowledge-based analysis	Gemei et al. 2013
Protein signature of 10 protein spots	Therapy response	Chemonaive OS tissues classified as good/poor responders	No	2D-DIGE and PCA	Kawai et al. 2008
Vimentin, tubulin-a1c, lamin B2, coatomer protein complex, epsilon subunit, zinc finger protein 133, ferritin light polypeptide, myosin, light chain 6, ezrin, transferrin, a1-antitrypsin, chaperonin-containing TCP1	Diagnosis	OS and benign bone tumor tissues	Yes, WB and IHC	2DE and PMF	Li et al. 2010
Peroxiredoxin-2	Therapy response	Chemonaive OS tissues classified as good/poor responders	Yes, WB	2D-DIGE and LC-LTQ ion trap MS/MS	Kikuta et al. 2010, Kubota et al. 2013
Heat shock protein 90 and clusterin	Prognosis	OS tissues from older adults and desmoid tumor tissues	Yes, TMA	LC-MS/MS	Rao et al. 2013
Serum amyloid A	Diagnosis	Plasma form OS and OC	Yes, WB	SELDI-ToF/MS	Li et al. 2006

(*continued*)

Table 4 (continued)

Proteins	Application	Biological samples	Validation	Technique	References
Serum amyloid A	Diagnosis	Serum from OS patients and healthy subjects	Yes, WB and ELISA	2D-DIGE and PMF	Jin et al. 2007
Cytochrome-c1	Diagnosis	Serum from OS patients and healthy subjects	Yes, WB	SELDI-ToF/MS	Li et al. 2009
Serum amyloid A and transthyretin	Therapy response	Plasma from OS patients before and after preoperative chemotherapy and classified as good/poor responders	Yes, WB	SELDI-ToF/MS	Li et al. 2011
Gelsolin (decrease)	Diagnosis	Serum from OS patients and healthy subjects	Yes, WB and ELISA	2D-DIGE and PMF	Jin et al. 2012
Two protein peaks at *M/Z* of 3954 Da and 6438 Da (not identified)		Plasma from OS, OC, and healthy volunteers	No	SELDI-ToF/MS	Gu et al. 2014

transformed cells. The overexpression of two of these, namely, ezrin and alpha-crystallin B chain, were confirmed by immune histochemistry or real-time PCR.

Recently, researchers in the field of experimental and clinical oncology have focused their attention on cancer stem cells (CSC). Several lines of evidences indicate that CSCs posses an elevated genotypic and phenotypic plasticity responsible for the heterogenicity of tumors and are involved not only in carcinogenesis but also in the metastatic process, invasion, therapeutic poor responsiveness, and recurrence of cancer. Although our comprehension of OS CSCs has notably improved, their role in OS pathophysiology is still largely unknown (Bernardini et al. 2014). To strengthen our knowledge of OS CSCs, global protein profiling can be extremely useful in uncovering their complexity as well as in selecting novel putative biomarkers. Several phenotypic changes were detected in two OS CSCs when compared to their parental cell lines (Table 1; Saini et al. 2012; Gemei et al. 2013). However, since none of these potential markers have been validated, their use as diagnostic or prognostic factors is still to be demonstrated.

Biomarkers from OS Tissues

Proteomic studies to identify specific OS protein markers were also conducted on tissue samples obtained from patients' biopsies (Kawai et al. 2008; Kikuta et al. 2010; Li et al. 2010; Kubota et al. 2013; Rao et al. 2013).

Li et al. compared the protein expression profile of malignant (osteoblastic, chondroblastic, and fibroblastic OS) and several benign (chondroblastoma, osteoblastoma, and giant cell) bone tumors using 2DE combined to PMF (Li et al. 2010). The overexpression in OS of two (TUBA1C and ZNF133) out of 12 upregulated protein spots was validated by WB and IHC and thus selected as potential OS biomarkers. Although authors did not extend the validation phase to normal bone tissue, these two proteins represent a starting point for the development of important molecular tools for OS diagnostic.

Analogously, a gel-based proteomic approach was carried out by Kondo to identify prognostic markers of OS responsiveness to chemotherapy (Kawai et al. 2008; Kikuta et al. 2010; Kubota et al. 2013). Authors detected the overexpression of peroxiredoxin 2 in OS tissue samples from chemonaive patients who were afterwards classified as poor responder to different chemotherapy protocols: combination of IFO, DOX, and CDDP (Kikuta et al. 2010) or combination of MTX, DOX, and CDDP (Kubota et al. 2013). The overexpression of peroxiredoxin 2 was further validated in a larger cohort of OS patients by WB and ROC analysis (AUC = 0.90, sensitivity = 83.3%, specificity = 85.7%, $p = 0.015$) that demonstrated the reliability of such a prognostic marker (Kubota et al. 2013).

Heat shock protein 90 and clusterin were found by a gel-free proteomic approach to be able to differentiate OS tissues from benign desmoid tissues (Rao et al. 2013). In particular, OS tissues were isolated from older adult patients with different background (Paget's disease, OS associated to dedifferentiated liposarcoma,

extraosseus OS, dedifferentiated periosteal OS) with the aim to define the protein profile related to a highly metastatic cancer.

Circulating OS Biomarkers

Finally, the occurrence of specific OS protein biomarkers was also explored in plasma samples.

Serum amyloid protein A (SAA) was found to be present in higher amount in OS patients than in osteochondroma patients (Li et al. 2006) or in healthy controls (Jin et al. 2007). Moreover, several authors demonstrated that SAA levels in OS patients might be used as marker to monitor relapses or response to chemotherapy (Jin et al. 2007; Li et al. 2011). Other OS plasmatic biomarkers include high level of cytochrome C as an early diagnostic indicator, while high level of transthyretin suggests a poor response to therapy (Li et al. 2009, 2011).

Potential Applications to Prognosis, Other Diseases, or Conditions

Osteosarcoma is a heterogeneous tumor. This is due to the lack of characteristic chromosomal translocations or alterations, the occurrence in different anatomical sites, and the presence of different histologic subtypes. This heterogeneity, in addition to biospecimens variability (tissues, cells, body fluids, etc.), and to the low incidence of the pathology, is reflected in post-genomics studies leading to nonoverlapping or discordant results and to a very challenging validation phase of potential biomarkers. Therefore it is likely to be difficult to identify genes, miRNAs, or proteins that could have reliable diagnostic and/or prognostic value in osteosarcoma. To overcome the problem related to the scarcity of clinical cases, the scientific and medical community should promote networks of biobanks by means of national and international reference centers. These networks should be committed to harmonize procedures and set common standards for biospecimens and clinical data collection and storage and to facilitate access to biological samples. A similar approach should be used with high-throughput approaches and comprehensive and integrated post-genomic investigations of patients should be required in order to overcome the intrinsic limitation of each related technology.

Our chance to understand the relationships between the individual molecular asset and the pathogenesis of disease, as well as the diversity of clinical outcomes or responses to therapies, will only be guaranteed by the use of high-quality biological samples with accurately phenotyped clinical data. This will likely lead to personalized medicines for OS patients.

Summary Points

- Osteosarcoma is the most common primary bone cancer in adolescents and young adults.
- The presence of metastatic disease at the time of diagnosis and responsiveness to chemotherapy are the principal prognostic factors for OS.
- There is an urgency for reliable biomarkers for early detection of OS, prediction of chemoresponsiveness, monitoring of treatment or relapses.
- "Omics" approaches identified biomarkers for several types of cancers as diagnostic/prognostic indicators.
- Novel diagnostic and prognostic biomarkers can also represent novel target for more effective and personalized therapies.
- Rarity of OS hinders the large and proper validation of biomarkers selected by post-genomics approaches.

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