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Biomarkers in Osteosarcoma

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Abstract

Osteosarcoma is the most common primary tumor of bone and accounts for approximately 19% of all malignant tumors of bone. It is the third most common malignant tumor in teenagers. More than twenty years ago, the advent of a multidisciplinary approach that combined multi-agent chemotherapy and limb-sparing surgery greatly improved the survival rate of patients with osteosarcoma. Unfortunately, since that time, survival rates have not dramatically improved. To date, the most powerful predictors of outcome have remained the ability to detect metastatic disease at diagnosis and the histopathologic response of the tumor to preoperative chemotherapy. Presently, 80% of patients who do not have distant metastases at initial diagnosis will become long-term survivors. Unfortunately, this means that approximately 20% of patients who do not present with metastases at diagnosis will not survive. This group of patients appears to be resistant to current treatment as attempts to intensify therapy after surgery for patients with a poor histopathologic response has not significantly improved survival rates. It is these patients that are in the greatest need of additional clinically relevant markers for prognosis and who can be most helped by molecular analysis. While steady progress has been made in the identification of genetic alterations in osteosarcoma, no individual molecular marker has thus far been demonstrated to have a better prognostic significance in the treatment of osteosarcomas than the current clinical markers. Thus there is clearly a need to employ new comprehensive analysis technologies to develop significantly more informative classification systems and to identify new therapeutic targets.

Keywords

Osteosarcoma; Chemotherapy; Biomarkers; High-throughput screening

Introduction

Osteosarcoma is the most common form of primary malignant bone tumor found in children and accounts for approximately 60% of the primary bone malignancies diagnosed within the first two decades of life. Osteosarcoma most often develops during periods of rapid skeletal growth with more than 50% of tumors occurring in the long bones of the appendicular skeleton.

Osteosarcoma is a histologically diverse tumor that arises from cells of osteoblastic lineage with many variations and mixed histology type tumors. This histological variation is likely a reflection of the differentiation state of the cells from which the tumor arose. Osteosarcomas can resemble all stages of osteoblast differentiation from the very primitive mesenchymal stem cell-like cells to the well-differentiated osteocytes. However, the hallmark characteristic of the disease is the production of osteoid by tumor cells. The disease typically presents as a highly aggressive neoplasm with frequent occurrence of distant metastases, typically in the lungs.

Osteosarcoma can occur in the intramedullary space or on the surface of the bone or, in rare cases, in other tissues of mesenchymal origin in the absence of bone (extraskelatal osteosarcoma). Plain radiographs, computed tomography, magnetic resonance imaging, angiography and dynamic bone scintigraphy are typically used for initial diagnosis and evaluation the extent of tumor involvement and the presence of metastases. The diagnosis must then be confirmed using open biopsy or fine needle biopsy for histological evaluation of the tumor. Although diagnosis can be made on the basis of fine needle aspirate, recent recommendations have been for a return to open biopsy to permit collection of sufficient material for both histological evaluation and biological studies ¹. As noted by these authors, progress in osteosarcoma research depends on the collection of sufficient tumor material to permit detailed molecular studies.

Treatment for osteosarcoma has followed a multidisciplinary approach. At present, standard therapy includes a four-drug neoadjuvant therapy consisting of high-dose methotrexate, doxorubicin, cisplatin and ifosfamide ² followed by limb-sparing surgery ³. This approach, which was pioneered in the 1980's, resulted in a significant improvement of survival for osteosarcoma patients. At present, patients with nonmetastatic osteosarcoma of the extremities have an expected 5-year survival rate of 70% ⁴. For patients with metastases at diagnosis or who had recurrence of osteosarcoma following initial disease remission, the 5-year survival rate has reached 20–30% ^{5, 6}.

Recent evidence has also suggested that the addition of liposomal muramyl tripeptide phosphatidyl ethanolamine to the treatment regimen enhanced event free survival (EFS) ². Unfortunately, chemotherapy for osteosarcoma remains one of the most arduous and exhausting therapies of any that is given for solid tumors and treatment of the primary tumor is associated with permanent disability to some degree in a significant proportion of patients ⁷. Moreover, there has been little improvement in patient outcome over the past twenty years. Even more critically, no standard, second line therapy exists for patients who relapse. Studies have shown that while further improvement cannot be achieved by dose intensification of treatment ^{8–10}, complications as a result of the intensified therapy have increased in frequency ¹¹, which suggest that new strategies are required. However, thus far, other drug regimens have been tried in relapsed patients with little effect. As a result of this need, a new international cooperative trial has opened in Europe and the United States. Ancillary biological studies associated with the ongoing European and American Osteosarcoma 1 trial (EURAMOS 1) are designed to explore new avenues with the promise of valuable biologic insights and rapid evaluation of investigational strategies applied to the treatment of osteosarcoma ⁷.

Historically, the most effective predictors of outcome in osteosarcoma have been clinical ¹². These have included the size of the tumor, the presence of metastases at diagnosis and the histopathologic response of the tumor to preoperative chemotherapy. Patients whose tumors show >90% necrosis upon definitive surgery following neoadjuvant chemotherapy have a much better five year survival rate than patients whose tumors do not respond to the chemotherapy ¹³. Serological markers such as alkaline phosphatase have been examined in patients with osteosarcoma and at least one study has found that normal serum levels of alkaline phosphatase at presentation had significantly longer times to recurrence when compared to high levels of serum alkaline phosphatase (25 months compared to 18 months) ¹⁴.

However, these clinical markers require that the patient first undergo the chemotherapeutic regimen with its associated short-term and long-term side effects ¹¹. Moreover, as many as 20% of patients are resistant to this treatment and attempts to intensify therapy after surgery for patients with a poor histopathologic response have not significantly improved survival rates ¹⁵. Thus the major challenges in osteosarcoma treatment today are: 1) identifying responders to current neoadjuvant therapy from non-responders prior to initiation of treatment. 2)

Providing alternative therapies for responders as well as non-responders. 3) Providing effective treatment for recurrent osteosarcoma. 4) Providing effective treatment for patients with metastatic disease at initial diagnosis. Each of these represents a unique challenge in biomarker detection. An examination of the steady progress in both the identification of genetic alterations in osteosarcoma and the advent of new comprehensive approaches to differentiate responders from non-responders is the purpose behind this review.

Biomarkers for Prognosis in Osteosarcoma

The need to identify patients that will or will not respond to standard therapy and the need to provide chemotherapeutic alternatives for these patients has fueled the effort to identify biomarkers that correlate with outcome in osteosarcoma. This effort has taken two directions – the examination of the role of single gene alterations in osteosarcoma and the use of high-throughput array-based technologies to develop signatures that predict poor survival outcome. Both approaches have also been used to try and identify novel targets for chemotherapy as well.

Individual genetic alterations in osteosarcoma

The analysis of the role of individual genes for prognosis in osteosarcoma has been confused with the study of their role in osteosarcoma initiation and progression. Many genes have been found to play a role in osteosarcoma tumorigenesis, however whether these same genes can be used to predict outcome is not always as simple to determine. The following examples show that some genes that are significantly involved in osteosarcoma may not be as useful in determining the outcome of the disease while others may play a highly significant role in predicting outcome.

The Retinoblastoma Susceptibility gene (RB1) in osteosarcoma

One of the earliest associations with osteosarcoma was in patients that survived bilateral retinoblastoma. Patients with bilateral retinoblastoma were found to have a significant risk for subsequently developing osteosarcoma. This increased risk occurred regardless of whether the patients received radiation treatment and the osteosarcoma tumors often occurred outside the field of radiation.

The discovery of the RB1 gene on human chromosome 13q14 allowed the discovery that bilateral retinoblastoma patients with osteosarcoma tumors shared a common mutation in the retinoblastoma and osteosarcoma tumors. This mutation, which was inherited in the patients as a heterozygous mutation, had frequently undergone a somatic loss of the remaining wildtype allele in the both the retinoblastoma and osteosarcoma tumors, making it a paradigm for tumor suppressor genes. The observation that osteosarcoma tumors, resected from bilateral retinoblastoma patients, underwent a loss of heterozygosity (LoH) in the same region of chromosome 13 that occurred in retinoblastoma tumors seemed to further solidified the relationship between these two diseases¹⁶. Furthermore, adolescent patients with osteosarcoma without bilateral retinoblastoma also had somatic homozygous mutations in the RB1 gene in the osteosarcoma tumors. These observations allowed different groups to show that mutations in RB1 occur in a high percentage of osteosarcomas^{17–21}.

Studies have shown that the RB1 gene acts in a variety of cellular functions including proliferation, differentiation and apoptosis. The RB1 protein binding pocket binds to a variety of target proteins including the E2F class of transcription factors and other proteins containing an LxCxE-box motif and inhibits their function²². The RB1 protein is regulated by phosphorylation by Cyclin Dependent Kinases CDK2, CDK4 and CDK6^{22–26}. This phosphorylation causes RB1 to release the bound proteins, which then are able to activate transcription. This phosphorylation of RB1 appears to be a critical event in the cell cycle

transition from the G1 vegetative growth phase to the S phase, proliferation and DNA replication phase ^{23, 25}.

As noted, loss of RB1 function appears to occur frequently in osteosarcoma tumors, and also to occur early in the tumorigenic process. Although initial efforts to correlate outcome with RB1 state did show correlation between RB1 status and survival ²⁷, more recent studies have failed to show a significant correlation ²⁸.

The TP53 pathway in osteosarcoma

Like Rb1, TP53 also plays a role in the cell cycle process. It is thought that p53 has a significant role in DNA repair by serving as a checkpoint after DNA damage. TP53 also is known to play a significant regulatory role in apoptosis ²⁹. Consequently, mutations in the TP53 gene are likely to lead to an inability to effectively respond to cellular DNA damage. Subsequent studies revealed that the TP53 gene is one of the most commonly mutated genes in human cancer ³⁰. Its pattern of loss in cancers suggests that it is another tumor suppressor paradigm. An association of TP53 with osteosarcoma was found in patients with Li-Fraumeni syndrome ^{31, 32}. Li-Fraumeni syndrome patients are identified as having two or more first-degree relatives with cancer before the age of 40, at least one of which is a sarcoma. Osteosarcoma is one of the most common tumors associated with Li-Fraumeni syndrome ³³, which led investigators to examine patients with the syndrome for inherited mutations in the TP53 gene ³⁴. They found evidence for inherited TP53 mutations in approximately 50% of the Li-Fraumeni families ^{35, 36}. Further study found that mutations in the TP53 gene occurred in a significant fraction of sporadic adolescent osteosarcomas ^{37, 38}.

The TP53 gene product, p53, controls a variety of cellular functions including DNA damage detection, DNA repair and DNA synthesis ³⁹. It also acts as a controller for apoptosis. When DNA is damaged either by UV or ionizing radiation or chemotherapy, the damage is sensed through a series of proteins including ATR, ATM, CAK, CHK1, and CHK2 that lead to activation of the p53 protein. The p53 protein then causes the cell to arrest until the cell can affect DNA repair. If the cell fails to repair the DNA damage, then the p53 protein initiates apoptosis resulting in cell death.

The p53 gene is regulated by a number of different genes. MDM2 binds p53, which not only inhibits its ability to arrest the cell cycle and induce apoptosis but also promotes its transportation from the nucleus to the cytoplasm for ubiquitination and degradation by the ubiquitin-mediated proteasomal protein degradation pathway ⁴⁰. ATR, ATM, CAK, CHK1, and CHK2 all act to phosphorylate p53 near its MDM2 binding region causing dissociation of p53 from MDM2 resulting in activation of p53. MDM2 is in turn regulated by p19^{ARF}, which is one of the gene products of the CDKN2A gene. MDM2 amplification has been observed in a significant fraction of osteosarcomas that do not have TP53 mutations suggesting that this is an alternate method of inactivating p53 function ^{41–43}.

Although TP53 plays a critical role in osteosarcoma tumorigenesis, studies examining the role of TP53 in predicting outcome for osteosarcoma have shown that p53 functional status is not a prognostic factor for response to chemotherapy ^{44, 45}. However, mutant p53 status showed some indication for association with decreased survival although the association was relatively minor ^{44, 46}.

Resistance to chemotherapy

Developing resistance to chemotherapy is a significant problem in patients with osteosarcoma contributing to disease relapse, progression, and death. Increased expression of human *glutathione S-transferase P1 (GSTP1)*, a phase II detoxification enzyme, has been associated

with a significantly higher relapse rate and a worse clinical outcome in osteosarcoma⁴⁷. Moreover, studies have found that *GSTP1* expression was upregulated in osteosarcoma cells when they were treated with doxorubicin or cisplatin and that upregulation of *GSTP1* caused the cells to become more resistant to doxorubicin and cisplatin⁴⁸. Conversely, suppression of *GSTP1* expression resulted in increased apoptosis and extensive DNA damage in response to doxorubicin and cisplatin. Taken together, these findings suggest that *GSTP1* is linked to prognosis through development of doxorubicin and cisplatin resistance in osteosarcoma.

A transmembrane protein known as P-glycoprotein facilitates the efflux of many chemotherapeutic agents including doxorubicin. Early studies had suggested that multiple drug resistance as a result of P-glycoprotein expression might be a prognostic factor in osteosarcoma^{49–52}. However, later evidence appears to show that while P-glycoprotein expression as determined by immunohistochemistry, did have correlation with disease progression, P-glycoprotein expression was not associated with the histologic response to combination chemotherapy regimens in patients with osteosarcoma⁵³. Furthermore, in a large prospective study (INT0133) P-glycoprotein expression in the biopsy specimen did not significantly increase the risk for adverse outcomes and did not predict outcome for osteosarcoma patients⁵⁴.

Chemokine receptors in osteosarcoma

Chemokines are small proteins involved with the trafficking of leukocytes by the interaction of cellular receptors⁵⁵. CXCR4 is a chemokine receptor that is specific for the chemokine CXCL12 (also known as stromal-derived factor 1 - SDF1). The combination of both receptor and ligand is thought to have an important role in the metastatic cascade in many types of cancer by creating a chemotactic gradient between the primary tumor site and the metastatic site⁵⁶. Several studies have found that CXCR4 is expressed by osteosarcomas^{57, 58} and that the expression level of CXCR4 is inversely correlated with overall survival and metastasis-free survival^{57, 59}. In an experimental mouse model, inhibition of CXCR4 resulted in a decrease in metastatic disease⁶⁰.

The role of ERBB2 in osteosarcoma

ERBB2, also known as HER2/neu, is proto-oncogene that is mapped to chromosome 17q11.2-q12. As a member of the epidermal growth factor receptor family, ERBB2 is more commonly known for its role in the tumorigenesis of breast cancer and as a major target for treatment in that disease. Under normal conditions, ERBB2 is a cell membrane bound receptor tyrosine kinase that is involved in signal transduction that lead to cell growth and differentiation. However, in breast cancer, receptor the status of ERBB2 in osteosarcoma has generated considerable controversy, with several laboratories reporting apparently conflicting results as to the significance of expression of ERBB2 in patient outcome^{61–75}. Reports of expression of ERBB2 in osteosarcoma vary from absent to overexpressed in 10–63% of cases. In osteosarcoma tumors, ERBB2 staining was frequently found to be diffuse and present mainly in the cytoplasm^{69, 72, 74}. This contrasts with what has been reported in breast cancer, in which erbB2 staining was increased but always localized to the plasma membrane^{76, 77}. However, at least one study of EGFR, ERBB2 and ERBB4 in osteosarcoma has found that all were constitutively phosphorylated when EGFR and ERBB2 were localized to an internal or non-surface location⁷⁴. Consistent with these observations of internalized erbB2, at least one study has found that Trastuzumab, the humanized antibody therapy directed against erbB2, was ineffective in osteosarcoma treatment⁷⁵. Finally, in breast cancer and other epithelial tumors, overexpression of ERBB2 is typically associated with genomic amplification of the ERBB2 locus, whereas in osteosarcoma genomic amplification of ERBB2 is rare^{64, 69}.

Ezrin, a metastatic determinant in osteosarcoma

The Ezrin or VIL2 gene encodes a cytoskeleton linker membrane protein that functions as a protein-tyrosine kinase substrate ⁷⁸. As a member of the ERM protein family, this protein typically serves as an intermediate between plasma membrane and the actin cytoskeleton. Specifically, regulation of ERM family proteins is typically due to phosphorylation or binding to PIP2. This action, in turn, exposes two binding sites. One site is used by an actin filament and the other is used by a transmembrane protein. Therefore, activation of ERM family proteins are thought to have a role in stabilizing cell surface proteins that are formed in response to extracellular signals. These interactions are thought to have characteristics important implications for metastasis, tumor progression, cell adhesion and migration ⁷⁹. Specifically, it is known to be involved in signal transduction of the AKT and the mitogen-activated protein kinase (MAPK) pathways of osteosarcoma ⁸⁰. When upregulated it has been shown that it can drive metastasis in mouse model osteosarcomas ⁸¹. It has also been shown that Ezrin can be used as a predictive marker for progression of osteosarcoma ^{82, 83}. Comparison of conventional high grade and low-grade osteosarcomas staining for Ezrin via immunohistochemical methods has shown that Ezrin immunoreactivity was absent in all low grade osteosarcoma samples but present in a significant portion of the high grade samples ^{83, 84}.

BIRC5 (Survivin) expression levels in metastatic osteosarcoma

Baculoviral IAP Repeat-Containing Protein 5, also known as Survivin, is a member of the Inhibitor of Apoptosis family of proteins. Survivin acts by binding to procaspase-3 and -7, which reduces the activity of these caspases and results in inhibition of cell death in cells exposed to apoptotic signals ⁸⁵. Survivin was found to be expressed in osteosarcoma tumors but not in normal tissues ^{86–89}. In one study, nuclear localization of survivin by immunohistochemistry was found to correlate with prolonged survival while cytoplasmic localization had no correlation with patient outcome ⁸⁶. Another study found nuclear and cytoplasmic staining in all osteosarcoma tumors but that expression of survivin correlated with increased malignancy ⁸⁸. Similarly, patients with metastatic osteosarcoma were found to have high levels of survivin expression in their initial biopsy specimens and there was a statistically significant difference in levels of expression between patients that had metastases and those that did not ^{87, 89}. Thus the effect of survivin on inhibiting apoptosis may have a role in osteosarcoma progression.

FAS and FASL in metastatic osteosarcoma

The FAS receptor and its ligand (FASL) belong to the tumor necrosis factor death receptor superfamily and are implicated in several types of primary malignancies and metastases ⁹⁰. In osteosarcoma, lung metastases were found to express low levels of FAS, while the primary tumors from the same patients often expressed high FAS levels ^{91–93}. This correlates with mouse models of osteosarcoma metastasis, which consistently showed an inverse relationship between FAS expression and metastatic potential ^{94, 95}. One possible model is that FASL is constitutively expressed in lung tissue and any FAS positive osteosarcoma tumor cells that entered the lungs would bind to the FASL and induce apoptosis ^{93, 95}. In osteosarcoma cell lines, induction of FASL by cyclophosphamide and its derivative ifosfamide mediates apoptosis in the osteosarcoma tumor cells via an autocrine paracrine loop by cross-linking with cell surface FAS ⁹⁶. IL-12 enhances the sensitivity of osteosarcoma cells to cyclophosphamide and its derivative ifosfamide by upregulating FAS ^{96, 97}. Confirming this model, treatment of mice with an aerosol form of gemcitabine resulted in increased FAS expression and subsequent tumor regression in several model systems ^{95, 98–100}.

Metalloproteinases and osteosarcoma metastatic potential

The matrix metalloproteinases (MMPs) are involved in the breakdown of the collagens of the extracellular matrix and as such play an important role in tissue remodeling and also cancer invasion and metastasis^{101, 102}. Increased expression of MMP-9 has been found to correlate with metastatic phenotype in osteosarcoma^{101, 103} while another study has also linked increased expression of MMP-1 with poor prognosis in osteosarcoma¹⁰⁴. Inhibitors of MMPs, such as TIMP-1 and RECK have been shown to inhibit invasiveness of osteosarcoma tumor cells in vitro and suggest that their levels of expression may also be correlated with metastatic potential in osteosarcoma^{102, 105}. Conversely, the urokinase plasminogen activator, uPA, which has been shown to upregulate MMP expression has been shown to have an inverse correlation between expression of uPA and metastasis in an animal model¹⁰⁶ and may be a candidate prognostic marker in human osteosarcoma.

Telomere maintenance and chromosomal instability as a prognostic factor in osteosarcoma

Telomere maintenance is regarded as a key mechanism in overcoming cellular senescence in tumor cells¹⁰⁷. During S phase DNA replication, small discrete units of DNA are lost from the ends of each chromosome. As the cells continue to divide, these chromosome ends become smaller, which eventually triggers cellular senescence¹⁰⁸. Two types of telomere maintenance mechanisms have been described in human tumors, telomerase activation and alternative lengthening of telomeres (ALT)¹⁰⁹. Although the vast majority of epithelial tumors rely on telomerase activation, osteosarcomas appear to more frequently rely on the ALT mechanism for telomere maintenance^{110, 111}. At least one study has found that absence of a functional telomere maintenance mechanism was associated with improved survival in osteosarcoma patients¹¹² suggesting that telomere maintenance may be an important aspect of osteosarcoma tumorigenesis. As an aside, it is curious to speculate on the karyotypic scrambling and chromosomal instability frequently seen in osteosarcomas and the role of telomerase maintenance, which at least in some models may regulate chromosomal instability^{113, 114}.

RECQL4 and Rothmund-Thomson syndrome

Studies of Rothmund-Thomson syndrome have linked osteosarcoma to gene mutations in RecQ protein-like4 gene (RECQL4) located at 8q24.3. Mutations in RECQL4 have been shown in a subset of Rothmund-Thompson patients^{115–117}. The RecQL4 protein has homology to the DNA helicase RecQ. The RecQ protein has been implicated in DNA double stranded break repair and unfaithful recombination [45,47]. Though the exact function of RECQL4 proteins remains unknown, DNA helicases function to aid in DNA replication, repair, and recombination. In patients with Rothmund-Thomson syndrome osteosarcoma occurs in patients with truncating mutations in RECQL4^{118–120}. In this select group of Rothmund-Thomson syndrome patients, the risk of developing osteosarcoma is 5%^{118, 121}.

The role of RECQL4 in osteosarcoma tumorigenesis and prognosis is problematic. One significant difference between RECQL4 and other gene in which inherited mutations predispose to osteosarcoma is that no somatic mutations of RECQL4 have been identified in sporadic cases of osteosarcoma¹¹⁹. This may reflect the fact that mutations in RECQL4 would only have an indirect effect on the cell. Thus the effect of RECQL4 mutations on prognosis is unlikely to be significant outside of patients with Rothmund-Thomson syndrome.

Other genes potentially linked to outcome in osteosarcoma

Along with Her2/neu, the WNT family of proteins is another growth factor family that has been implicated as candidate biomarkers in osteosarcoma. Commonly known for their role in normal skeletal limb formation and tumorigenesis¹²², the WNT family proteins bind to cell surface receptor proteins in the Frizzled family (FZD). As a result of this initial binding,

Disheveled family proteins are activated. This activation of the Disheveled protein (DSH) inhibits 15 a protein complex, which includes axin, GSK-3, and APC, from promoting the proteolytic degradation of signaling molecule B-catenin¹²³ resulting in increased levels of cytoplasmic B-catenin. B-catenin has the ability to translocate across the nuclear membrane and interact with the T cell factor/Lymphoid enhancer factor (TCF/LEF) family of transcription factors, which promote the transcription of specific WNT-responsive genes¹²³. Some of these genes are known to be associated with matrix degrading enzymes, cell cycle regulators, and tumor metastasis in osteosarcomas^{122, 124–126}. LDL receptor related protein 5 (LRP5) has also been proposed as a prognostic marker for high-grade osteosarcomas¹²⁷.

High-throughput analysis methods in osteosarcoma

The completion of the sequencing of the human genome has accelerated efforts to examine chromosomal abnormalities including large-scale amplifications, deletions and copy number variations in various types of cancer. This type of analysis has been facilitated by the availability of high-throughput array-based technologies that can scan the entire genome with high resolution for such genomic variations¹²⁸.

Comparative Genome Hybridization and Copy Number Analysis

A number of groups have used comparative genome hybridization to study osteosarcomas^{129–141}. Their analyses confirmed that osteosarcomas have a chromosomal instability phenotype with numerous chromosomal alterations. Despite the high frequency of alterations, there were several common chromosomal alterations detected. Chromosomal gains were detected at 1p, 5p, 6p, 8q and 17p. Chromosomal losses were observed at 2q, 10p, 14q, 15q, and 16p. Regions of chromosomal amplification were found at 1q21-q22, 1p34-p36, 5p13-p15, 6p12-21, 12q12-q14 and Xp11.2 with the most common amplifications at 8q23-q24 and 17p11.2 – p12, which were detected in a majority of tumors. The only common region of subchromosomal deletion was at 18q21-q22. In all cases, amplifications outnumbered deletions. Because of small sample sizes, none of the chromosomal regions correlated with clinical outcome.

cDNA Microarray Analysis

Gene expression analysis by cDNA microarray has been used as a comprehensive analysis technique to develop more informative classification systems and to identify new therapeutic targets. It allows researchers to analyze the gene expression of thousands of genes in a single experiment. These arrays result in a comprehensive survey of the expression patterns in the tumors, which can in turn be used to identify molecular pathways and potential molecular targets for diagnosis and treatment. In theory, microarray analysis can be used to develop genomic expression signatures that can distinguish outcome and response to therapy as well as divide tumors into molecularly defined categories and associations with specific genetic pathways that may suggest novel therapeutic approaches. Unfortunately, microarrays remain a challenge for the clinic due to issues with specimen collection and heterogeneity that can complicate analysis. Moreover, microarray results typically require validation by a complementary technique supported by strong bioinformatics data analysis techniques to be interpreted.

Several laboratories have done microarray analysis on osteosarcoma tumor samples. Several laboratories used osteosarcoma tumor cell lines^{142–147} or mouse models¹⁴⁸ and focused on known target pathways to examine the perturbations in those systems. Other analyses have focused on the clinical question of identifying patients that will or will not respond to chemotherapy^{149–151}. In the latter analyses, although each research group identified a robust genetic expression signature identifying chemotherapy-resistant pediatric osteosarcomas there was no overlap between the genes in the three signatures. The only correlation between the

three results was that it appeared that most of the genes in the each of the signatures showed higher expression in the tumors with a poor response to chemotherapy. The significance of this discovery is uncertain at present however, the analysis clearly demonstrates the need for more analysis to develop a consistent signature capable of predicting response to therapy.

Other high throughput methods of analysis in osteosarcoma

Other high throughput technologies have yet to be applied to osteosarcoma prognosis. Initial studies of SNP-genotyping have identified risk factors for developing osteosarcoma¹⁵², but as yet have not been applied to prognosis and outcome studies. Other high throughput technologies such as exon arrays and high throughput sequencing techniques such as 454 and Solexa sequencing have yet to be applied to the question of osteosarcoma prognosis, although such studies are now in the works.

Expert Opinion

Where is the future of osteosarcoma biomarker research? Currently, patients with localized disease have a 5-year survival rate that is at least 70% however patients with metastatic or recurrent disease have less than 20% chance of long-term survival despite aggressive therapies^{5, 6}. These outcomes have not changed significantly in over 20 years. Thus, as we have shown, much of the focus on osteosarcoma biomarkers has been on the categorization of patients who will or will not respond to current chemotherapeutic regimens. This will of course have a significant benefit for the patients' quality of life as patients who are identified as likely to respond to standard therapy may be able to receive and respond to a lower dose of the treatment regimen than is currently offered. Likewise, patients who are likely to not respond to the current therapy may be offered alternative therapies based on biological targets identified as part of the biomarker discovery process. The future of osteosarcoma treatment must involve a more molecular approach to the measurement of relevant clinical prognostic factors and the development of treatments based on the molecular profile of tumors at diagnosis with adjuvant therapies directed towards dysfunctional molecular pathways rather than the use of broad cytotoxic chemical strategies.

A second focus is on the nature of the metastatic phenotype in osteosarcoma. The discovery of the roles of Ezrin and FAS/FASL signaling may be important in understanding this critical aspect of osteosarcoma progression. Analysis of the function of these genes in lung metastases may result in the discovery of ways to preventing the spread of micrometastases to the lungs.

Finally, one aspect of osteosarcoma biology that is becoming important is the role of chromosomal instability and telomere maintenance. The advent of studies in copy number analysis has shown that there are areas of specific amplification and deletion that may harbor important genes in osteosarcoma tumorigenesis. Moreover, maintaining chromosomal telomeres has also been shown to have an important role in cancer and in preventing senescence^{109, 153}. Telomere maintenance may also be an important aspect of osteosarcoma tumorigenesis and may be linked to prognosis¹¹².

The future of high throughput analysis in osteosarcoma research

The frontier of osteosarcoma outcome research is now largely in the province of high throughput methods of discovery. As we have shown, comparative genome hybridization and copy number analysis have yielded important new discoveries that may affect osteosarcoma treatment. Microarray analysis has been useful as a tool of discovery although the robustness of the signatures that have been identified is still in question. Another potentially useful high throughput screening method will be mass spectrometry and the ability to analyze protein profiles and generate proteomic signatures. Initial efforts in proteomic analysis of

osteosarcoma have been exciting^{154–156} and may lead to more clinically robust analyses that genomic signatures.

Looming in the future is the use of high throughput sequencing to analyze genetic changes directly in the DNA of tumors. As has been shown in breast and colon cancer, direct high throughput sequencing of the genomes can detect commonly mutated genes as well as those genes that are mutated at lower frequency but still constitute important determinants in the tumorigenic phenotype^{157, 158}. Other potentially promising high throughput studies include metabolomics and the study of the kinome as functional aspects of osteosarcoma tumor biology.

Osteosarcoma is a fascinating and complex disease, encompassing many different biological and clinical distinctions that have complicated the treatment and outcome of this disease. Despite its rarity, it has been important in the understanding of cancer and has played a role in the discovery of many of the important genes in cancer. The need for improvement in treatment is a clear call for new therapeutic targets and more clinical trials. The new cooperative trial jointly supported by European and North American study groups⁷ is a exciting start in this direction. The combination of new trials and new methods of analysis promises to open this difficult disease to new avenues of care.

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