The progress of molecular diagnostics of osteosarcoma

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1. ABSTRACT

Despite significant advances in the diagnosis and treatment of osteosarcoma in recent years, overall survival has remained low for over 2 decades. The standard diagnosis of osteosarcoma requires a combination of clinical presentation, radiologic studies, and pathologic tissue evaluation. A typical “Codman’s triangle” in radiologic evaluation is vital in making correct diagnosis for middle or late stage of osteosarcoma. However, there is an actual demand for novel molecular markers with high sensitivity and stability for the diagnosis of early events of osteosarcoma and also the probability of recurrence and metastasis. Except that, some highly relevant gene mutations with these events could also provide valuable information regarding osteosarcoma protection. In this review, we will focus on the molecular markers which have been discovered in recent years with potential application of early stage and recurrence diagnosis and protection.

2. INTRODUCTION

Osteosarcoma displays a bimodal distribution with two peaks in the late adolescent and young adult period and after the 60's of life, respectively (1). Many evidences indicate an association between rapid bone growth and osteosarcoma, as an earlier age of onset of osteosarcoma in female adolescent and early adult than male counterparts and patients with osteosarcoma significantly taller than general population have been discovered (2,3). Moreover, 56% osteosarcomas occur around metaphyseal location of the knee (4).

The initiation of osteosarcoma is not quite clear. One possibility could be the radiation induced accumulative genetic mutation. Because it was noted that there was a link between radiation exposure and osteosarcoma in female radium dial worker (5). High incidence of osteosarcoma in children treated by radiotherapy for solid tumor is also observed (6). During the progression stage, tumor cells will undergo uncontrolled proliferation through somatic mutation of some oncogenes such as AP-1 and tumor suppression genes such as p53 and retinoblastoma (Rb) genes (7,8). Then the proliferative osteosarcoma cells will interplay with local osteoblast and osteoclast through cytokines such as IL-6, IL-11, et al. to promote bone resorption by activating osteoclast (9,10). Meanwhile, osteosarcoma will degrade bone matrix through secreting MMP-2/ MMP-9, and undergo epithelial to mesenchymal (EMT) transition to facilitate their metastasis to surrounding soft connective tissue or long distance organ such as lung (11-13).

Therefore, based on the knowledge of the pathogenesis of osteosarcoma, it is necessary to find the genes and molecules including proteins and microRNAs which can regulate the proliferation and dormancy of tumor cells.

3. GENETIC REGULATION

Osteosarcoma is characterized by a high level of chromosomal instability (CIN) (14). CIN can be further categorized into numerical CIN (N-CIN) and structural CIN (S-CIN). N-CIN leads to copy number alterations of chromosomes, while S-CIN leads to chromosomal rearrangements, breakages, and mutations.

Maintenant, the major discovered mutations in osteosarcoma are TP53, RB1, and RECQL4. TP53 mutation has been discovered significantly correlated with high levels of genomic instability in osteosarcoma (15).
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Because p53 has important role in DNA repair, so its inactivated mutation will cause genomic instability. This genomic instability will cause other mutations. And if some mutations of cell cycle related genes happen, it will initiate the cancer. For example, in 2008, Seth et al. discovered that double deletion of pRb and p53 in osteoblast precursors (Osx1 Cre) can cause more severe osteosarcoma than p53 single deletion (16). However, one thing is noteworthy that in pRb single deletion mice, they have much lower ratio of osteosarcoma than p53 single knockout mice. It indicates that the proposed model of second mutation initiated tumorigenesis due to p53 inactivation does not work well in pRb case. Furthermore, this mechanism of this synergistic effect of p53 and pRb needs to be clarified.

The next issue is that if p53 is a good marker for clinical prognosis. However, some results are conflicting, and most of them are inconclusive or sample size is not big enough. For example, in Tsuchiya’s study in 2000, they discovered that in 21 osteosarcoma patients, the event-free survival (EFS) was worse with TP53 alterations than without TP53 alterations (17). In another study in 1997, Papai et al. discovered a direct correlation between mutated p53 proteins and resistance to therapy based on their histological analysis of biopsy (18). However, in a 2004 meta-analysis study, it was shown that TP53 status is not associated with the histologic response to chemotherapy, but it may be associated with decreased survival (19). Two very recent meta-analysis also support this point of view that p53 is an effective biomarker of survival in patients with osteosarcoma (20,21). However, it was shown in a multicenter study of 196 patients that p53 mutations cannot predict for metastasis in patients with high-grade osteosarcoma (22). Therefore, the mechanism how mutated p53 cause poor prognosis of osteosarcoma needs to be further investigated.

RB1 (retinoblastoma protein 1) is a major inhibitory regulator of the G1 to S phase progression in the cell cycle through its binding to E2F after its phosphorylation by cyclin dependent kinase 4 (CDK4) (23). pRb not only regulates the cell proliferation, its deficiency can also inhibit the osteoblast differentiation of osteoprogenitor. (24) But the mechanism how pRb regulates this differentiation is still not very clear. It is discovered in another recent basic study that pRb deficiency leads to the N-cadherin loss and enhanced migration by an indirect consequence (25).

In clinic, the expression levels of both pRb and cyclin D1 have a clear correlation with clinical outcome, suggesting that these parameters could be used as prognostic markers (26). But the relation between pRb mutation and clinical metastasis of osteosarcoma is still unknown.

RECQL4, a DNA helicase, has been implicated in DNA replication, DNA repair and recombination, and transcription of RNA (27,28). As RECQL4 gene product can suppress genetic recombination and ensure accurate chromosome segregation, its somatic mutation is frequently relevant with Rothmund-Thomson syndrome (RTS) related osteosarcoma (29,30). However, the specific mechanism is also not very clear.

There are also some other gene mutations involved in osteosarcoma. Mouse double minute 2 homolog (MDM2), located in 12q13-15, can mediate TP53 ubiquitination and degradation through its E3 ubiquitin ligase activity. It has been proved that MDM2 gene amplification is highly associated with tumor progression and metastasis in osteosarcoma, but not in primary osteosarcoma (31). Other two recent studies reveal that COPS3, another component of the proteasome pathway targeting p53, has increased copy number in osteosarcoma (32,33).

4. SIGNALING REGULATION

Although genetic regulation contributes a lot to tumorigenesis of osteosarcoma, it is not easy to diagnose it based on genetic analysis because sometimes the genetic mutation happens in a somatic manner. Therefore, to check some important molecules in certain activated signaling pathways could be helpful as another way of osteosarcoma diagnosis.

Wnt signaling has an essential role in regulating bone formation and remodeling during embryonic development and fracture repair. Its abnormalities give rise to several pathological bone conditions, including abnormal bone mass, osteosarcomas and bone loss in multiple myeloma (34). The role of Wnt signaling in osteosarcoma is also a hot field. There are at least 4 Wnt signaling pathways have been extensively investigated: the canonical Wnt/β-catenin pathway, and the non-canonical Wnt/Ca²⁺ pathway, Wnt/planar cell polarity (Wnt/PCP) pathway, and Wnt/protein kinase A (Wnt/PAK) pathway (35). The canonical Wnt pathway is initiated by the binding of appropriate Wnt ligands to the Fzs and LRP-5/6 co-receptor, and then the activated receptor complex will inhibit the degradation of β-catenin through the activation of intracellular protein, Dishevelled (Dvl) and inhibition of GSK-3β. The degraded β-catenin will then accumulate and translocate to the nucleus to be in concert with members of the T cell factor/lymphoid enhancer factor (TCF/LEF) and activate the transcription of a wide downstream genes including c-myc and cyclin D1 (36).

In osteosarcoma, overexpression of Wnt ligands and Frizzled and LRP co-receptors are very common. For example, LRP-5 overexpression is significantly involved in osteosarcoma disease progression and metastasis. (37) A dominant-negative soluble LRP-5 can block the invasiveness of Saos-2 cells through reversing
the epithelial-to-mesenchymal transition (EMT) and together with reduced expression of metalloproteinase (MMP) 2 and 14. (38) The same group also proved the anti-tumor function of dominant-negative soluble LRP-5 in vivo through an orthotopic xenograft model (39). As Wnt signaling pathway is tightly controlled by secreted antagonists that either bind Wnt receptors or directly bind Wnt ligands, these antagonists and Wnt proteins could be good indicators and even therapeutic reagents for osteosarcoma. Wnt inhibitory factor 1 (Wif-1), the secreted frizzled-related protein (SFRP) family, was recently shown to be epigenetically silenced due to promoter hypermethylation (40). Dickkopf (Dkk) family proteins (Dkk-1, Dkk2, and Dkk-3) that bind with high affinity to LRP-5 or LRP-6 are proved to have opposite functions in the progress and metastasis of osteosarcoma and Ewing sarcoma. Dkk-1 and Dkk-2 have pro-metastatic function and their elevated expression levels have been detected in both paediatric patients and mouse models (41,42). On the opposite, Dkk-3 can suppress tumorigenic potential and pulmonary metastasis in an orthotopic xenograft model of osteosarcoma (43). The expression level of β-catenin could also be a good indicator for the diagnosis of osteosarcoma and its lung metastasis, as its expression level is correlated with the invasiveness of osteosarcoma, and chemical inhibition of the Wnt/β-catenin signaling enhanced MTX mediated death of Saos-2 cells (44).

Except the canonical Wnt signaling pathway, the non-canonical Wnt signaling pathway also play important roles in osteosarcoma, though such investigations are not as many as canonical Wnt signaling pathway. Wnt5a is one commonly studied non-canonical Wnt ligand. Wnt5a and its co-receptor ROR2 expression level correlates with disease severity in osteosarcoma patients (45). Furthermore, this cell-autonomous cycling manner of Wnt5a signaling pathway has been proved to enhance the migration of osteosarcoma cells through several mechanisms including upregulation of chemokine receptor CXCR4 (46), upregulation of matrix metalloproteinase (MMP-13) (47), and improvement of EMT transition (48). For another non-canonical Wnt protein, the Wnt11, there is until now only 1 publication showing that Wnt11 does not express in 4 human osteosarcoma cell lines including U2OS, HOS, 143B, and Saos-2 (37). CD99, a 32kDa highly glycosylated transmembrane protein generally present in osteoblasts but lost in osteosarcoma, can increase contact strength and reactivate stop-migration signals through inhibiting c-Src and ROCK2 activity and recruiting N-cadherin and β-catenin to the adherens junctions (38). The downregulation of surface expression of CD99 could be a diagnostic marker for osteosarcoma.

The Notch pathway is a highly conserved regulatory signaling network involved in many developmental processes and several cancers. Abnormal activated Notch pathway can promote metastasis of osteosarcoma (49). Engin F, et al. discovered significant upregulation of Notch1 and Osterix in human osteosarcoma cell lines Saos-2 and primary human osteosarcoma tumor samples. Moreover, gamma-secretase inhibitors or dominant negative Mastermind-like protein (DN-MAML) can decrease osteosarcoma cell proliferation in vitro (50). However, in another study in the same year by Tanaka M, et al., they only discovered overexpression of Notch2 in the biopsy specimens using the same real-time PCR method; while the Notch1 expression is downregulated (51). Although the role of Notch1 and Notch2 in osteosarcoma needs further investigation, the common discovery in both studies of the inhibitory effects of gamma-secretase inhibitors on the growth of osteosarcoma cells indicates that Notch signaling pathway is convincingly involved.

Hedgehog, a transmembrane receptor which is important for cell-cell conjunction, has been recently proved to be involved in osteosarcoma progression and metastasis. High expression levels of the Hedgehog ligand gene, IHH, and target genes, PTCH1 and GLI1 are detected in most high-grade human osteosarcoma samples (52). GLI2 overexpression is also detected in human osteosarcoma biopsy. Its overexpression promotes mesenchymal stem cell proliferation and accelerated their cell cycle progression (53). Some hedgehog signaling pathway inhibitors have been proved to inhibit osteosarcoma progression and metastases. For example, cyclopamine targeting receptor Smoothened to inhibit Hedgehog pathway has been proved to inhibit osteosarcoma pulmonary metastases (54). By inhibiting GLI proteins, the ancient drug arsenic trioxide has been proved able to prevent osteosarcoma growth (55).

5. miRNA REGULATION

MicroRNA (miRNA) was initially identified in Caenorhabditis elegans in 1993(56). Numerous studies have demonstrated their important regulatory roles in tumor growth and migration in different types of tumor (57-60). A number of recent studies also demonstrated the important roles of miRNA in osteosarcoma (61). Basically, according to their pathological functions, these miRNAs can be categorized into three groups: proliferation-related miRNAs, metastasis-related miRNAs, and chemotherapy-related miRNAs.

In the proliferation-related miRNAs, some act as oncogene which is overexpressed in osteosarcoma, while some others act as tumor suppressor gene which is underexpressed. For example, a recent study revealed a combination of miRNA signatures for osteosarcoma diagnosis which include high expression of miR-181a, miR-181b, and miR-181c as well as reduced expression of miR-16, miR-29b, and miR-142-5p (62). However, the mechanism how these miRNAs work is still not clear.
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miR-214 functions as an oncogene in osteosarcoma through direct inhibition of leucine zipper putative tumors suppressor 1 (LZTS1) (63). MiR-27a can function as an oncogene by targeting MAP2K4, which in turn inhibits cell proliferation and migration through the JNK/p38 signaling pathway (64). The overexpression of miR-802 is discovered in osteosarcoma tissues compared with adjacent normal tissues, and enforced expression of miR-802 is able to promote cell proliferation in U2OS and MG63 cells. During this process, p27, a negative cell-cycle regulator, is negatively regulated by miR-802. However, if this regulation is direct or not is still unknown (65). miR-128, a direct inhibitor of PTEN, is proved to enhance osteosarcoma cells MG63 and U2OS proliferation by activating PTEN/Akt signaling pathway (66). Generally speaking, overexpressed molecules are much easier to be used as diagnostic markers than underexpressed molecules. However, much more miRNAs have inhibitory effects on proliferation in osteosarcoma cells, and their downregulation will promote the tumor cells to expand. For example, downregulation of miR-3928 can promote osteosarcoma growth by targeting ERBB3, IL-6R, and CDK6 (67). Through targeting Rho-associated protein kinase 1 (ROCK1), miR-145 can inhibit osteosarcoma cell proliferation and invasion. (68,69) By using miRNA microarray to compare the human osteosarcomas with normal human skeletal muscle, miR-133a and miR-133b expression level are found 135 folds and 47 folds decrease respectively. Overexpression of miR-133b in U2OS and MG-63 osteosarcoma cell line by stable transfection can inhibit cell proliferation, invasion and migration by targeting Akt and FAK signaling pathway (70). Loss of miR-132 can predict poor prognosis in patients with primary osteosarcoma by directly suppressing cyclin E1 (71,72). MiR-542-3p, a p53 positive regulator, which stables p53 protein and inhibits cell proliferation, is downregulated in osteosarcoma (73). It could also be a diagnostic marker for osteosarcoma.

Except the proliferation-related miRNAs, there are also a lot of metastasis-related miRNAs in osteosarcoma. They will promote the invasion, migration, and metastasis of tumor cells. For example, Osaki M. et al. discovered the downregulation of miR-143 correlates with the lung metastasis of human osteosarcoma cells probably via MMP-13 upregulation. But how miR-143 regulate MMP-13 is not indicated in this study (73). Furthermore, exosome-formed synthetic miR-143 is used to transfer the osteosarcoma cells, and proved to reduce the migration of osteosarcoma cells (74). MiR-218 can inhibit osteosarcoma cell migration and invasion by down-regulating MMP2 and MMP9 (75). Although there have been already numerous studies on the regulation of EMT by miRNA, it is still blank of such studies on osteosarcoma. For example, in colon cancer, loss of miR-101 expression promotes Wnt/b-catenin signaling pathway and EMT (76). Ectopic overexpression of miR-374a promotes EMT and metastasis of breast cancer both in vitro and in vivo (77).

There are also some miRNAs which can regulate the resistance of osteosarcoma cells to chemotherapy. It has been found that in chemoresistant osteosarcoma samples, miR-33a is up-regulated and can down-regulate TWIST expression to inhibit the apoptosis inducing effect of TWIST. In MG63 cells, overexpression of miR-33a significantly decreases cisplatin-induced cell apoptosis (78). In the osteosarcoma tumor xenografts treated with chemotherapeutic agents (including doxorubicin, cisplatin and ifosfamide), miR-140 is identified high expression level. The chemotherapy resistant mechanism is p53 dependent and HDAC4-mediated (79). MiR-215, through the suppression of dentineless protein homolog (DLT), induces a decreased cell proliferation by causing G2-arrest, leading to an increase in chemoresistance to MTX and TDX of osteosarcoma (80).

Circulating miRNA is the easiest molecule for diagnosis, however until now not too many circulating miRNAs are discovered to be ideal for the diagnosis of osteosarcoma and its metastasis. In 2013, Ouyang L, et al. defined a three-plasma miRNA signature as novel, non-invasive biomarker for osteosarcoma diagnosis. They found there was higher level of circulating miR-21, and lower levels of miR-143 and miR-199a-3p in the serum of osteosarcoma patient. Circulating miR-21 and miR-143 expression correlated with both metastasis status and histological subtype; while, miR-199a-3p levels only correlated with histological subtype (81). A more recent study in 2014 by Tian et al revealed that plasma miR-34b was causally associated with osteosarcoma risk and related with its metastatic status, because miR-34b level in both plasma and local tissue are decreased in osteosarcoma patients (82).

6. CIRCULATING TUMOR CELL MARKER

Circulating molecules or cells are usually ideal targets for diagnosis due to their easier manipulation compared with the local molecules or cells. However, it is sometimes hard to find a circulating molecules or cells which are highly correlated with the specific disease. Sometimes, the content of the specific markers are too low in the circulation to be detected. Therefore, the sensitivity and specificity are the key issues for these markers.

Very recently, Li group discovered cell-surface vimentin (CSV) as an exclusive marker on sarcoma circulating tumor cells, which has high sensitivity and specificity (83). The same group also claimed in 2013 that they developed a new antibody 84-1, which can
specifically target epithelial-mesenchymal transformed (EMT) circulating tumor cells with high sensitivity, though the studies for the characterization of the epitope for 84-1 are still ongoing (84). As early as in 2000, a very interesting investigation tried to isolate the circulating tumor cells and made PCR reaction to detect osteosarcoma. In their results, 91% (10 of 11) osteosarcoma patients have significantly higher type I collagen mRNA level compared with healthy subjects (85).

7. CONCLUSIONS AND PERSPECTIVES

The diagnosis of osteosarcoma is undergoing major changes, from the traditional “Codman’s triangle” evaluation method based on clinical presentation, radiologic studies, and pathologic tissue evaluation to the modern molecule-based way of evaluation. The original purpose of the investigation of these novel molecular markers is to help the diagnosis of the initiation of
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Osteosarcoma, the prevention of the metastasis of late stage osteosarcoma, and the recurrence of dormant tumor cells. In general, these novel markers can come from 3 levels: the gene, the protein, and the miRNA. It is already known several gene mutations could be correlated with osteosarcoma such as P53, RB1, and RECQL4. However, sometimes these kinds of mutation are not easy to be detected, as these mutations only happen in somatic tumor cells. Therefore, it is more important to find some important and specific molecules involved in the tumor progression. There are many signaling pathways involved in the control of tumor cell proliferation. For example, the canonical and non-canonical Wnt/β-catenin signaling pathway (Summarized in Figure 1), and the Akt signaling pathway have been proved related to the proliferation of osteosarcoma. Moreover, the Wnt/β-catenin signaling pathway and the Notch signaling pathway have been proved related to the metastasis of osteosarcoma. However, there is also the same problem as the genetic marker has that these signaling pathway is only activated in the local lesion or the metastasis lesion. As a new field of investigation, the role of miRNAs as novel markers for diagnosis has been paid more attentions than genetic markers and signaling markers. And also novel miRNAs are found highly correlated with the progression and metastasis of osteosarcoma. (Summarized in Table 1) However, the limitation of ways of detection still exists.

The more ideal way of diagnosis would be to find some circulating markers such as miRNA, cytokine, or other small molecules, or even sometimes the cells. However, such kind of efforts is far from satisfactory. There is still a long way to go in this field.

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Figure 1. A. The canonical Wnt pathway. In the presence of Wnt ligands, the binding of Wnt to the receptor Fz and co-receptor LRP-5/6 leads to the inhibition of GSK-3β, which results in the intracellular accumulation of β-catenin and its nuclear translocation. Intracellular β-catenin then activates the transcription of a wide range of downstream genes including c-myc and cyclin D1 to promote tumor cell proliferation. B. The non-canonical Wnt pathway. In the presence of Wnt-5a, the binding of Wnt-5a to the Fz receptor and ROR-2 co-receptor can activate intracellular calcium level and PKC. Also it can trigger the activation of the small GTPases Ras homolog gene family (eg. RasA) and Rac. Activation of RasA leads to the activation of the Ras-associated kinase ROCK. Rac activation stimulates c-Jun N-terminal Kinase (JNK) signaling cascade.
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Abbreviations: Rb: retinoblastoma; EMT, mesenchymal; CIN: chromosomal instability; N-CIN: numerical CIN; CDK4: cyclin dependent kinase 4; RTS: Rothmund-Thomson syndrome; MDM2: Mouse double minute 2 homolog; Wnt/ PKA: Wnt/protein kinase A; Dvl: Dishevelled; TCF/LEF: T cell factor/lymphoid enhancer factor; MMP 2: metalloproteinase 2; Wif-1: Wnt inhibitory factor 1; SFRP: secreted frizzled-related protein; Dkk: Dickkopf; DN-MAML: dominant negative Mastermind-like protein; miRNA: MicroRNA; LZTS1: leucine zipper putative tumors suppressor 1; ROCK1: Rho-associated protein kinase 1; CSV: cell-surface vimentin

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