# Genetics of Osteosarcoma

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**Abstract**

Osteosarcomas (OSs) are functionally defined tumors, and the function may be affected by two endogenous factors: cell-of-origin and genetic alterations. Since the identification of mutations of two tumor suppressor genes, RB1 and p53, a large number of studies have been conducted to identify genetic alterations of OS. Finally the whole-genome sequencing analyses provided us with the landscape of genetic alterations of OS, which confirmed the previously known features of OS such as anomalous structural variations. In addition to p53 and RB1, ATRX and DLG2 genes were identified as candidates of new driver genes in OS. Animal models provided us with the role of each driver mutation. Therefore now we are able to illustrate the basic molecular machineries to drive OS cells. However, it seems that the additional genetic alterations may endow OS cells with phenotypic heterogeneity and also tools to protect them from the molecular target therapy, which should be considered for the development of new therapeutic modalities.

**Keywords**

RB1 • p53 • Mutation • Whole-genome sequence • Gene expression profile

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T. Ueda, A. Kawai (eds.), *Osteosarcoma*, DOI 10.1007/978-4-431-55696-1_1

## 1.1 Introduction

Osteosarcoma (OS) is defined as a primary malignant tumor with mesenchymal cells producing osteoid and/or bone [1, 2]. Thus OS is a tumor defined by its function, although the level of function may be variable, because the amount of osteoid and/or bone differed considerably among tumors, ranging from extensive mature bone formation to minimum amount of immature osteoid [1, 2]. As for the cell-of-origin of OS, cells on the osteogenic lineage in bone marrow stromal cells are reasonable candidates, which include various types of cells [3]. Also it is well known that OS tumor cells exhibit karyotypes with a high degree of complexity, which may contribute to the biological heterogeneity [4]. Therefore it is reasonable to speculate that OSs diagnosed under the current criteria cover various tumors with heterogeneous cellular and genetic background. Despite such factors potentially contributing biological heterogeneity, clinical features of OS are remarkably homogeneous, in terms of their aggressive phenotype and response to chemotherapy, suggesting the common molecular pathway for the development of this type of tumors [5]. Investigation for molecular genetics of OS started when the mutation of retinoblastoma gene (RB1) and p53 gene was found in OS in 1986 and 1987, respectively [6, 7]. Since then a large amounts of efforts have been devoted to understand OS at the molecular level [8]. Recent progresses in the field of genome analyses and animal models gradually have disclosed the molecular architecture of OS. The general feature is, however, not remarkably different from what we have expected after the discovery of RB1 and p53 mutation in OS, and still we need a progress to understand this ominous disease. In this chapter, by reviewing the progress in the past decades, the road to be followed is discussed.

## 1.2 Genetic Alterations of OS

There are two types of genetic alterations in any type of cancers: driver mutations and passenger mutations [9]. The driver mutations are causally related to the development of each type of tumors and indispensable for the principle features of tumor cells endowing unlimited growth ability, invasiveness, and escaping from apoptosis. In contrast, the passenger mutations are simply accumulated over the course of development and cell growth and may be redundant and dispensable. Although these definitions are clear, it is not easy to distinguish between them from the information obtained by classical genetic analyses. Recent innovations in whole-genome analyses using next-generation sequencing machines, however, have opened a novel world of cancer genetics and made it possible to overlook the landscape of genetic events in each type of tumors [10, 11]. Based on the complete information of genetic alterations such as structural variations (SVs), copy number variations (CNVs), and single-nucleotide variations (SNVs), driver mutations for each cancer can be identified by sophisticated mathematical analyses [9]. In the case of OS, two reports were published using next-generation sequencing

**Table 1.1** Genetic alteration identified by the next-generation sequencing analyses

| Reference | [12] | [13] |
|---|---|---|
| No. of samples | 34 primary tumors | 10 primary tumors |
| Type of samples | Whole-genome sequencing | Whole-exome sequencing |
| Total number of mutation/tumor | 1483.1 | NA |
| No. of mutations in exon | 1017 | 202 |
| No. mutations in exon/tumor | 25.5 | 15.5 |
| No. mutated genes | 932 | 195 |
| No. of mutated genes in common | 19 | |
| No. of mutated genes found in more than two tumors | 20 | 1 |

technology, of which one performed the whole-genome sequencing (WGS) [12] and the other described the whole-exome sequencing (WES) [13] (Table 1.1).

### 1.2.1 General Features of Genetic Alterations

#### 1.2.1.1 Structural Variation (SV)

It is well known that karyotype of OS exhibits extreme aneuploidy with a large number of aberrant chromosomes [4]. The chromosomal instability of OS cells was also demonstrated by the frequent occurrence of loss of heterozygosity (LOH) [14]. In agreement with these previous data, the WGS by Chen et al. showed a high incidence of SVs in OS [12]. The study identified 10,806 SVs in 34 tumors, of which 377 produced in-frame fusion genes. RNA-sequencing data were available for 64 predicted fusion SVs and among them 15 fusion genes were expressed. Using the previously reported data of other tumors (embryonal rhabdomyosarcoma, acute T cell lymphocytic leukemia, and medulloblastoma), the basal mutation rate and the number of SNVs, SVs, and CNVs of OSs were compared with those of these tumors, and the number of SVs was significantly higher in OS compared with other tumors. LOH was also detected with high frequency; 10 out of 13 tumors showed more than 7 LOH, indicating again the high incidence of SVs in OS. Chromothripsis, literally "chromosome shattering," is a recently discovered phenomenon by which thousands of clustered chromosomal rearrangements occur by a single mutation event in the localized and confined genomic region [15]. The WGS of OS found four cases showed chromothripsis in the region of chromosomes 6q, 13q, 14q, and 17q in each case.

#### 1.2.1.2 Single-Nucleotide Variation (SNV)

The WGS of 34 OS samples by Chen et al. identified 1483.1 SNV/tumor, and this frequency ($1.15 \times 10^{-6}$) was comparable with the standard mutation rate in the human genome [12]. Among these SNVs, 25.2 mutations/tumor (ranging 5–103) resulted in either missense, nonsense, or splicing mutations, and the total number of

genes showing these types of mutations were 932. Among them 20 genes were found in more than two cases, but only four genes (*p53*, *RB1*, *ATRX*, and *DLG2*) were identified by the statistical analyses as significantly mutated genes, and these genes were described as driver mutations in the next section.

Joseph et al. performed the WES using ten primary samples and three cell lines [13]. The number of mutations in the exon regions resulting in amino acid changes was 195, and surprisingly only one gene (the *p53* gene) was mutated in more than two cases. Therefore the p53 gene was the only gene which showed mutations in more than two cases both in the WGS and WES studies. The difference between two studies may be caused by the difference in the sequencing method, but may reflect the heterogeneity of OSs.

### 1.2.2 Driver Mutations

As described in the previous section, the WGS isolated four genes as the driver mutation of OS, of which two (the *RB1* and *p53* gene) were previously recognized as the driver mutation because of their involvement of hereditary cases and the result of mouse models, and the remaining two genes (the *ATRX* and *DLG2* gene) were novel candidates for the driver mutation of OS.

#### 1.2.2.1 The *p53* Gene

The involvement of p53 in OS was first demonstrated by somatic mutations [7], and LOH on the chromosome 17p [16], and further confirmed by the identification of its mutant as the causative for a familial cancer syndrome, Li-Fraumeni syndrome, which is characterized by a high risk for various cancers including breast, brain, and adrenal grand cancers and osteosarcomas [17, 18]. The p53 protein is responsible for monitoring the integrity of the genome and the control of cell cycle checkpoints after DNA damage [19]. The number of mutations in the *p53* gene identified by the WGS was 28/34 (82.5 %), in which 19 were SVs and 9 were SNVs. Although some tumors were free from the *p53* gene mutation, mutations of genes directly regulating the p53 such as the *MDM2* gene were found in such tumors [20], and therefore it is acceptable to consider that almost all OSs have abnormalities in genes on the p53 pathway.

The driver function of mutant p53 in osteosarcomagenesis was further confirmed by animal models (Table 1.2). p53 knockout mice were fertile and developed a number of tumors including osteosarcoma [21]. Conditional knockout mice using the expression of genes in osteogenic lineages such as the *Prx1*, *Osterix*, and *Col1A1* genes developed OS with a high frequency, almost 100 % in some cases [22–26]. Although the precise mechanism of how the loss of p53 can induce OS so frequently is not yet known, it might be related to the function of p53 as a guardian of genome [19], because the high incidence of SVs is the hallmark of OS.

**Table 1.2** Tumor incidence of genetic engineered mice

| Predicting target cells | Cre-driver gene | Target gene | Predicted genotype | Incidence of OS | Reference |
|---|---|---|---|---|---|
| Mesenchymal stem cells | Prx1 | p53 and RB1 | $p53^{-/-}$ | 61 % | [22] |
| | | | $p53^{-/-}$:$RB1^{-/-}$ | 18 % | |
| | | p53 and RB1 | $p53^{-/-}$ | 62 % | [23] |
| | | | $p53^{-/-}$:$RB1^{+/-}$ | 92 % | |
| | | | $p53^{-/-}$:$RB1^{-/-}$ | 29 % | |
| Pre-osteoblast | Osterix | p53 and RB1 | $p53^{-/-}$ | 87.8 % | [24] |
| | | | $RB1^{-/-}$ | 0 % | |
| | | | $p53^{+/-}$:$RB1^{+/-}$ | 50 % | |
| | | | $p53^{+/-}$:$RB1^{-/-}$ | 90 % | |
| | | | $p53^{-/-}$:$RB1^{+/-}$ | (207 days) | |
| | | | $p53^{-/-}$:$RB1^{-/-}$ | (147 days) | |
| | | p53 and RB1 | $p53^{-/-}$ | 100 % | [25] |
| | | | $RB1^{-/-}$ | 0 % | |
| | | | $p53^{+/-}$:$RB1^{+/-}$ | 30.0 % | |
| | | | $p53^{+/-}$:$RB1^{-/-}$ | 77.8 % | |
| | | | $p53^{-/-}$:$RB1^{+/-}$ | (292 days) | |
| | | | $p53^{-/-}$:$RB1^{-/-}$ | (127 days) | |
| | Col1A1 (3.6 kb) | p53 | $p53^{-/-}$ | 60 % | [26] |
| Osteoblast | Col1A1 (2.3 kb) | p53 | $p53^{-/-}$ | 85 % | [22] |
| | | Notch | Exogenous NICD | 100 % | [63] |
| | Osteocalcin | Ptc | $Ptc^{+/-}$:$p53^{+/-}$ | 70 % | [65] |

#### 1.2.2.2 The *RB1* Gene

Retinoblastoma (RB) is a malignant tumor that develops in the eyes of infants, and approximately 25 % of patients show bilateral and multiple tumors, which are caused by germline mutations of the *RB1* gene [27]. The *RB1* gene is a ubiquitously expressed gene, the encoded protein of which regulates the cell cycle through the control of cyclins [28]. The loss-of-function mutations of *RB1* induce abnormal cell growth, and therefore this gene is called a tumor suppressor gene [28]. Patients with germline mutations of the *RB1* gene have a high risk of developing other malignant tumors during their lifetime, with OSs most frequently encountered [29]. The

mutation search in sporadic OS revealed a loss of functional RB protein in approximately 60 % of sporadic OSs, suggesting that RB1 plays a critical role in the development of not only RB but also OS [30]. Thus the *RB1* gene is the first gene mutated in OS with a high frequency, although it is not yet clear why the loss of RB protein predisposes the high risk of OS.

The WGS by Chen et al. discovered ten mutations of the *RB1* gene among 34 cases, of which seven were SVs and three were SNVs [12]. The frequency of mutation was lower than those of the p53 gene, but the function of RB1 was also inhibited by mutations of RB-associated genes such as the amplification of the CDK4 [31] and cyclin D [31] genes and the functional loss of the p16 gene by promoter methylation [32], and therefore the loss of RB function is also an important driver mutation in OS.

The mice model story of the RB1, however, was not as simple as in the case of the p53 (Table 1.2). Simple knockout mouse of the RB1 gene was embryonic lethal, and the heterozygous mice, which represented hereditary patients of RB, developed pinealomas but not retinoblastomas or osteosarcomas [33]. As same as the p53 gene, several lines of conditional knockout mice have been generated using the expression of genes on the osteogenic lineages. In contrast to the *p53* gene, the loss of RB1 in these cells failed to produce OS in most of cases [24, 25]. The effect of loss of RB1 was only manifested when these mice were crossed with p53 knockout mice, in which loss of RB1 accelerated the tumor formation and reduced the survival time [24, 25].

It is not yet known that why the loss of RB1 preferentially induces osteosarcomas, even though the function of RB1 is important in any types of cells. One of the hypothetical explanations is that RB1 protein has some specific roles in osteogenic differentiation. However, conflicting results were reported by in vitro and in vivo studies as for the effect of loss of RB1 on osteogenic differentiation [34–36]. This issue should be further investigated to understand precise role of RB1 mutations in the development of OS.

#### 1.2.2.3 The *ATRX* and *DLG2* Gene

The mutations of the *ATRX* (ATP-dependent helicase ATRX) gene was found in 10/34 cases (five as SV and five as SNV) [12]. ATRX is involved in ALT (alternative lengthening of telomere) [37], which is the main mechanism for the maintenance of the telomere length in sarcomas [8]. The mutations of *DLG2* (disc, large homolog 2) was found in 18/34 cases, all of which were SVs [12]. DLG2 is a member of the membrane-associated guanylate kinase family with multiple PDZ domains and involved in epithelial polarity during cell division [38]. In *Drosophila*, DLG is a tumor suppressor, but the tumor suppressor function was not yet confirmed in human cancers.

### 1.2.3 Genes Involved in the Hereditary Predisposition

#### 1.2.3.1 DNA Helicase Genes

DNA helicases consist of family of enzymes catalyzing the separation of double-strand DNA in several cellular processes such as DNA replication and DNA repair [39]. There are several hereditary diseases caused by the mutation of DNA helicase genes including Bloom syndrome (caused by the mutation of the *RECQL2* gene) [40], Werner syndrome (*RECQL3* or *WRN* gene) [41], and Rothmund-Thomson syndrome (*RECQL4* or *RTS* gene) [42]. Patients with genetic defects in these genes manifest a number of disorders and are predisposed to cancers including OS [39]. Among these helicase genes, mutations of the *RECQL4* gene seem to be most closely linked with the development of OS [43]. The important differences between the *RB1* or *p53* genes and DNA helicase genes in terms of the involvement of OS are that the former genes were frequently mutated in sporadic cases as somatic mutations, whereas almost no somatic mutations have been reported in the latter genes [44]. Although the hereditary involvements of DNA helicase genes are clearly observed in human cases, no definite observation was found in mice models of these genes. Homozygous *Recql4* mutant mice developed variable phenotype depending on the type of mutations, but the incidence of OS development was very low [45]. Therefore, although the extreme aneuploidy of OS suggested the link between the DNA repair systems and tumor development, it is not yet known how the mutations of DNA helicase genes were involved in the development of OS.

#### 1.2.3.2 Single-Nucleotide Polymorphism Associated with OS

The genome-wide association study (GWAS) has been performed to find genetic factors contributing the development of each disease in various fields including sarcomas [46, 47]. In the case of osteosarcoma, two SNPs were found to be associated with the risk for the development of osteosarcoma [48]. One of them is in the *GRM4* gene that encodes a metabotropic glutamate receptor, which involves c-AMP signaling cascade. The glutamate signaling is best characterized in the central nervous system, and its role in the bone metabolism is not known, although bone tissues expressed the GRM4 gene [49]. The effect of identified SNP for the regulation of the GRM4 gene is also not known. However, from the standpoint of recent focus in cancer research, the identification of a gene involving metabolic pathway as a risk factor is an interesting matter. The expression of the GRM4 gene is expressed in OS cells [50] and is associated with aggressive phenotypes of several cancers [51, 52]. Functional analyses of the GRM4 gene in OS cells may provide a key to answer this association.

### 1.2.4 Genes on the Signal Pathways Involved in OS

Studies of the molecular mechanisms of growth and progression in OS have identified more than 20 genetic alterations (Fig. 1.1). Most of them, however,

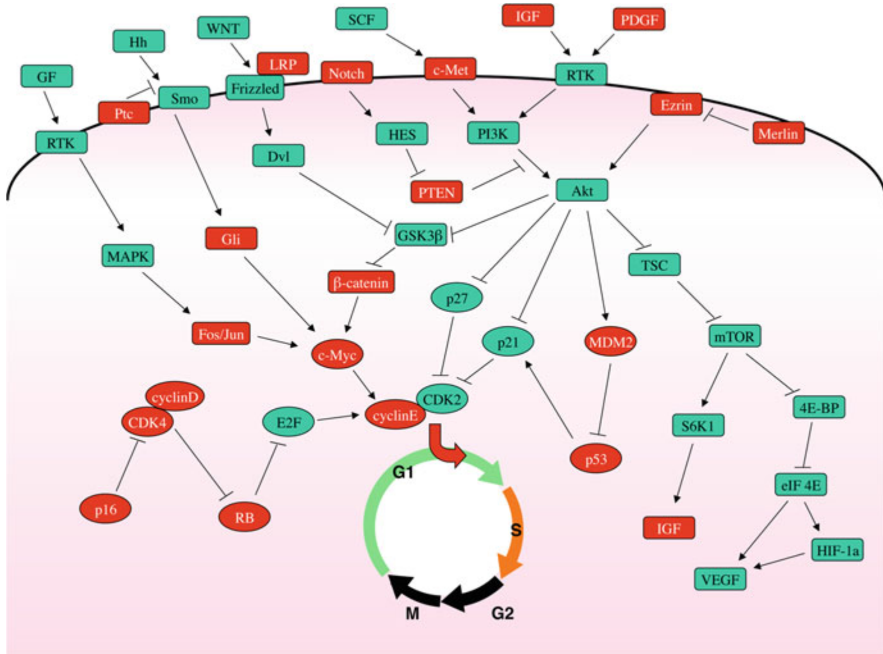


**Fig. 1.1** Genetic alterations in osteosarcoma. Genes with mutations (DNA and/or RNA level) are indicated with *red*

were dysregulation in mRNA or protein level, and few mutations were found in genomic DNA level. The most striking feature was their redundancy in the growth signals. OSs expressed the receptors for IGF, VEGF, HER2, ErbB-4, PTHR, and HGF, and many of them are redundant [53–59]. This imposed the difference in the development of molecular target therapy for OS. One of typical examples was the recent clinical trial of mTOR inhibitor. Several signal pathways on OS are connected to the Akt kinase through the activation of PI3K. Activated Akt then activates mTOR via inhibition of TSC, which then activates S6K and eIF4E, resulting in the activation of invasion-related protein such as VEGF. Activated Akt also inhibits the function of GSK-3, resulting in the nuclear accumulation of β-catenin, which then drives target such as c-myc. These data suggested that Akt is a hub molecular for growth signaling in OS, and the inhibition of mTOR function seemed to be a promising molecular approach to the treatment of OS. The result of clinical trial using novel mTOR inhibitors, however, showed minimum responses possibly due to the activation of other signal pathways [60]. Although this illustrated the difficulty to apply the molecular target therapy for OS, several clinical trials using chemical targeting following signal pathways are currently ongoing.

#### 1.2.4.1 The Notch Pathway

The Notch pathway is one of the evolutionally conserved pathways and manifests various functions in the development and homeostasis [61]. Involvement of the Notch pathway in OS was reported in several studies, in which the Notch signal was upregulated in tumor samples and the inhibition of this pathway suppresses tumor cell activity [62]. Transgenic mice containing the activating domain of Notch (Notch intracellular domain, NICD) driven by the *Col1A1* promoter developed human OS-like tumors with complete penetrance, and combination with p53 knockout mice accelerated the tumor formation [63]. Although no definite mutations were found in molecules on the Notch sigal pathway, inhibition of this pathyway will be one of therapeutic targets.

#### 1.2.4.2 The Hedgehog Pathway

Hedgehog signal is known to be involved in the human OS, although no definite genomic alteration on this signal pathway is found [64]. Patched is the repressive receptor of Hedgehog signal, and the loss of patched resulted in the acceleration of Hedgehog signal. Homozygous knockout mice of the *Patched* gene was lethal, and heterozygous mice develop OS when there were crossed with $p53^{+/-}$ mice [65].

#### 1.2.4.3 The Wnt Pathway

The Wnt signal is one of the critical signal pathway for the development, maintenance, and regeneration of bone tissues [66]. Nuclear accumulation of the beta-catenin protein in OS was reported [67], and the inhibition of WNT signal suppressed the aggressive phenotype of OS cell lines [68].

## 1.3 Expression Profiles of OS

In addition to the genomic information, the information of gene expression profiles of tumors have been used to identify genes for predicting features of tumors such as the aggressive phenotype and the sensitivity for chemotherapeutic drugs.

### 1.3.1 Prediction for Aggressive Phenotype

Several groups have tried to evaluate gene expression profile of OS to elucidate the specific features of OS. Leonard et al. compared gene expression profile of OS with those of mesenchymal stem cells, their putative precursors, and isolated several genes expressed highly in OS, one of which was the Ezrin encoding a cytoskeleton-associated molecule [69]. The Ezrin gene was also identified in the independent study of canine OS [70]. Khanna et al. compared the gene expression profiles of canine OS with and without distant metastasis and identified the Ezrin gene as metastasis-predicting gene [70]. Because the Ezrin activates Akt, which in turn activates mTOR signal, the clinical trials have been conducted as described in the

previous section [60]. Although the data was negative, the gene expression profiling is one of the powerful strategies to isolate the phenotype-related genes.

### 1.3.2 Prediction for Drug Sensitivity

It is a general agreement that the response to neoadjuvant chemotherapy is the most reliable prognostic factors, and therefore the identification of genes predicting the sensitivity for each chemotherapy provides important information for selecting the strategy. Searching literatures identified four articles regarding this issue [71–74]. All of them used basically similar protocols consisted of MTX, CDDP, and ADR, and the number of samples were comparable (Table 1.3). The response ratio of each study showed similar results, and each study identified a number of genes up- and downregulated in poor responders. Unfortunately, very few genes were commonly up- or downregulated among four studies, and no particular signal pathways were detected to be involved in the chemosensitivity. This may be caused by the difference in the platform of expression analyses, the preparation of samples, and/or the method for the evaluation but also may reflect biological heterogeneity of OS. Interestingly, the genes detected as commonly up- or downregulated were those related to basic cellular metabolisms. NAD(P)H dehydrogenase genes were identified as the only gene upregulated in all four studies, and the upregulation of the hydroxyacyl-coenzyme A dehydrogenase gene was identified three of four studies. Considering the redundancy in growth signal pathways in OS, these results suggest that basic components of metabolic process might be better targets for future therapies. Larger samples with a unified chemotherapeutic protocol may be required to answer this question.

## 1.4 Conclusion

Accumulation of genetic, molecular, and cellular information on OS enables us to illustrate the hypothetical processes during osteosarcomagenesis (Fig. 1.2). Cell of origin may be broad among cells on several steps in the osteogenic differentiation. Apparently the loss of function of p53 gene is the key driver mutation, and the most important role of mutant p53 in OS tumorigenesis may be induction of chromosomal instability. Osteogenic property of tumors cells should not be inhibited, because the production of osteoid/bone is critical to be diagnosed as OS and the loss of RB function may contribute to accelerate the tumorigenesis with maintaining the osteogenic property. Therefore the loss of function of these two factors may be indispensable events in OS. The function of two newly identified genes, *ATRX* and *DLG2*, in OS may add new insights of molecular signature of OS. Therefore now we are able to illustrate the basic molecular machineries to drive OS cells. However, from the therapeutic view point, the redundancy of growth signal of OS is a tough obstacle to be overcome. OS cells seem to be driven in the automatic mode without the control of driver, of which the content may be different

**Table 1.3** Identification of genes predicting response for chemotherapy

| References | [71] | [72] | [73] | [74] |
|---|---|---|---|---|
| No. of samples | 13 | 30 | 20 | 20 |
| Type of samples | Pre-Cx biopsy | Post-Cx resection | Post-Cx resection | Pre-Cx biopsy |
| Content of Cx | CDDP, DPX, MTX, IFO | CDDP, DOX, MTX | CDDP, DOX, MTX | CDDP, DOX, MTX, IFO |
| Response (good:poor) | 6:7 | 15:15 | 7:13 | 9:11 |
| No. of probes | 23,010 | 12,625 | 9216 | 5776 |
| No. of predicting genes | 60 | 99 | 45 | 44 |
| No. of genes upregulated in poor cases | 48 | 59 | 41 | 28 |
| No. of genes downregulated in poor cases | 12 | 40 | 4 | 16 |

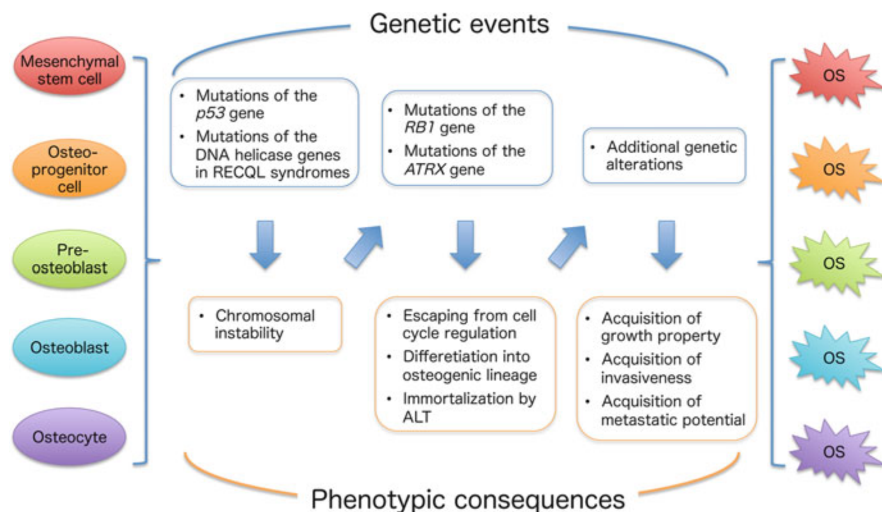


**Fig. 1.2** Hypothetical consequence of the development of osteosarcoma

in each tumor. If it is the case, the future therapeutic will be either general approach to shut off the energy of tumor cells targeting the molecules of basic cellular metabolism or super-personalized therapy based on bioinformatics on each tumor. Because of the limitation of space, the recent topics in cancer research such as epigenetic abnormality and micro-RNA were not covered in this chapter, both of which may have important roles in OS, and should be discussed in the next opportunity.

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