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RESEARCH

# Fuzzy Clustering Analysis of Osteosarcoma Related Genes

**Kai Chen · Dajiang Wu · Yushu Bai · Xiaodong Zhu · Ziqiang Chen · Chuanfeng Wang · Yingchuan Zhao · Ming Li**

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**Abstract** Osteosarcoma is the most common malignant bone-tumor with a peak manifestation during the second and third decade of life. In order to explore the influence of genetic factors on the mechanism of osteosarcoma by analyzing the inter relationship between osteosarcoma and its related genes, and then provide potential genetic references for the prevention, diagnosis and treatment of osteosarcoma, we collected osteosarcoma related gene sequences in Genebank of National Center for Biotechnology Information (NCBI) and local alignment analysis for a pair of sequences was carried out to identify the measurement association among related sequences. Then fuzzy clustering method was used for clustering analysis so as to contact the unknown genes through the consistent osteosarcoma related genes in one class. From the result of fuzzy clustering analysis, we could classify the osteosarcoma related genes into two groups and deduced that the genes clustered into one group had similar function. Based on this knowledge, we found more genes related to the pathogenesis of osteosarcoma and these genes could exert similar function as Runx2, a risk factor confirmed in osteosarcoma, this study may help better understand the genetic mechanism and provide new molecular markers and therapies for osteosarcoma.

**Keywords** Osteosarcoma · Gene sequences · Fuzzy clustering · Runx2

Kai Chen and Dajiang Wu contributed equally to this work.

K. Chen · D. Wu · Y. Bai · X. Zhu · Z. Chen · C. Wang · Y. Zhao · M. Li (✉)
Department of Orthopedics, Changhai Hospital, Shanghai 200433, China
e-mail: minglil@hotmail.com

## Introduction

Osteosarcoma, predominantly targets the adolescent age group [1], is the most common primary sarcoma of bone, representing about 35 % of cases and constituting approximately 0.07 % of all neoplasms [2]. Although the long-term survival rate have increased in the past few years with newly devised chemotherapy regimes, the prognosis is still poor. With the development of molecular biology for tumor, concept of gene therapy for tumor is proposed and the experimental results demonstrate good potential for its clinical application [3].

Recent studies have suggested that diverse genetic alterations in osteosarcoma cells such as gain or loss of chromosomes, mutation in tumor suppressor genes, epigenetic modifications and specific pathways may play important roles in the pathogenesis of osteosarcoma [4]. However, the molecular mechanisms underlying the initiation, development and metastasis of osteosarcoma are still not very clear. Therefore further understanding of osteosarcoma biology is urgently needed so as to optimize treatment strategies, identify molecular markers and develop new chemotherapeutic drugs.

A number of biomarkers such as vascular endothelial growth factor (VEGF) [5], microvascular density (MVD) [6], the retinoblastoma (Rb) gene [7], pigment epithelium derived factor (PEDF) [8] have been studied and identified for their roles played in the pathogenesis of osteosarcoma. In addition, it is worthy to mention that Runx2, a central regulator of skeletal development, which can induce cell cycle arrest and bone specific gene expression, leads to osteoblast and chondrocyte differentiation and maturation [9, 10]. Runx2 deficiency or mutation can cause severe bone abnormalities and osteosarcoma in mouse and human [11]. This study aims to analyze the genes relate to osteosarcoma by fuzzy clustering method and study the influence of genetic factors on the pathogenesis of osteosarcoma, so as to

provide new strategies for the prevention, clinical diagnosis and treatment of osteosarcoma.

## Materials and Methods

### Resources of Data

The osteosarcoma-related gene mRNA sequences of rat (Rattus norvegicus) were researched from the Genebank database of the National Center for Biotechnology Information (NCBI) of the USA with keyword "osteosarcoma". Among which, 21 recorded mRNAs were chose for further analysis, and the gene names in the database were *IL6, JUN, MAFG, MAPK3, NFYA, OS9, OSM, PRKCD, PRKG2, RB1, CASP3, RUNX2, SPP1, STAT3, CDKN2A, ENDOG, EP300, FOS, FOSB, GFAP* and *IBSP* respectively.

## Results and Analysis

In this study, fuzzy clustering method was selected to analyze the data. Fuzzy clustering is one technology that can classify the objective things through the establishment of fuzzy similarity relation based on their characteristics, the degree of closeness and similarity [12], which has been widely used in many areas of science and technology and the results are good [13].

The sequences of group A were assigned to the variable name: A={X1, *X*2, X3, X4 ..... X21, X22}, wherein X1 represented the sequence of *IL6*, *X*2 represented the sequence of *JUN*, and the rest could be deduced from this. We performed pair-wised sequence local alignment by using CLUSTAL W [14] and BLAST [15, 16]. Herein the local alignment of a pair-wises sequences referred to the one-to-one correspondence relationship of the respective character between the two sequences. A score value of the similarity of two sequences was given by matching, substitution, insertion or deletion of a character. Finally, a score matrix X=(Xij) 22 * 22 (i, j=1, 2, ....., 21, 22) was obtained by aligning partly the sequences of group A two by two (Fig. 1).

We established a fuzzy relation matrix for the scored matrix using the maximum and minimum method [17] in the fuzzy relationship, that was to characterize the similarity between the objects in group A described above and (where i=1,2, ....., 21) by using number rij ∈ [0, 1]. The detailed calculation formula for the establishment of rij (i, j=1,2, … 21) by maximum and minimum method was as follows:

$$R_{ij} = \mu(xi, xj) = \frac{\sum_{k=1}^{n} \min(x_{ik}, x_{ij})}{\sum_{k=1}^{n} \max(x_{ik}, x_{ij})}$$

Then the fuzzy relation matrix R=(rij)$_{21*21}$ was established after taking the data of the scored matrix into the calculation formula mentioned above (Fig. 2).

As we could see from the Fig. 2, this matrix satisfied the following properties: (1) $R_{ii}$=1 (i=1,2, ..... 21), which mean R=(rij) $_{21 * 21}$ was a reflexive matrix; (2) $R_{ii}$=rij (i, j=1,2, … 21), which mean R=(rij) $_{21 * 21}$ was a symmetric matrix. After that, we classified the fuzzy relation matrix R=(rij)$_{21*21}$ by the principle of direct clustering in fuzzy diagram. The concrete analysis process was as follows, the element (rij=1,2, ..... 21) in group A was connected sequentially in descending order from the matrix R=(rij), if there was a step loop emerging, just skipped that step until all the subjects were connected. The interconnected elements with the given connection strength threshold α not less than rij were classified as one class. According to this, a segmentation tree diagram for different threshold α was constructed as shown in Fig. 3.

| | x1 | x2 | x3 | x4 | x5 | x6 | x7 | x8 | x9 | x10 | x11 | x12 | x13 | x14 | x15 | x16 | x17 | x18 | x19 | x20 | x21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| x1 | 2. 565 | | | | | | | | | | | | | | | | | | | | |
| x2 | 1. 872 | 2. 159 | | | | | | | | | | | | | | | | | | | |
| x3 | 2. 565 | 1. 718 | 2. 747 | | | | | | | | | | | | | | | | | | |
| x4 | 2. 565 | 2. 411 | 2. 747 | 2. 970 | | | | | | | | | | | | | | | | | |
| x5 | 2. 411 | 2. 159 | 2. 970 | 2. 159 | 3. 258 | | | | | | | | | | | | | | | | |
| x6 | 2. 565 | 1. 872 | 2. 159 | 1. 959 | 0. 719 | 2. 970 | | | | | | | | | | | | | | | |
| x7 | 2. 411 | 2. 411 | 2. 747 | 3. 258 | 2. 159 | 1. 872 | 3. 664 | | | | | | | | | | | | | | |
| x8 | 2. 277 | 2. 054 | 2. 054 | 2. 411 | 2. 747 | 2. 411 | 2. 277 | 2. 565 | | | | | | | | | | | | | |
| x9 | 1. 872 | 2. 565 | 2. 970 | 2. 411 | 2. 277 | 2. 411 | 2. 277 | 2. 747 | 3. 258 | | | | | | | | | | | | |
| x10 | 2. 565 | 2. 411 | 2. 411 | 3. 258 | 1. 718 | 2. 277 | 2. 054 | 2. 565 | 2. 411 | 3. 258 | | | | | | | | | | | |
| x11 | 1. 959 | 1. 718 | 1. 872 | 2. 411 | 1. 792 | 1. 649 | 1. 584 | 2. 411 | 2. 159 | 2. 277 | 2. 411 | | | | | | | | | | |
| x12 | 2. 277 | 2. 747 | 2. 054 | 2. 747 | 2. 277 | 2. 565 | 2. 565 | 2. 747 | 2. 565 | 2. 054 | 2. 277 | 3. 664 | | | | | | | | | |
| x13 | 1. 872 | 2. 054 | 2. 159 | 3. 258 | 2. 970 | 2. 411 | 2. 277 | 3. 258 | 1. 959 | 2. 159 | 2. 747 | 1. 959 | 3. 258 | | | | | | | | |
| x14 | 2. 565 | 2. 054 | 2. 054 | 1. 959 | 2. 277 | 2. 565 | 1. 959 | 1. 959 | 1. 792 | 2. 747 | 1. 792 | 2. 565 | 2. 411 | 2. 747 | | | | | | | |
| x15 | 2. 054 | 2. 277 | 2. 159 | 2. 277 | 2. 054 | 1. 792 | 1. 523 | 1. 718 | 2. 411 | 2. 277 | 2. 159 | 2. 277 | 2. 277 | 2. 054 | 2. 565 | | | | | | |
| x16 | 3. 258 | 2. 565 | 2. 411 | 2. 411 | 2. 970 | 2. 747 | 2. 411 | 2. 970 | 2. 277 | 2. 277 | 2. 565 | 2. 970 | 3. 258 | 2. 747 | 2. 747 | 3. 664 | | | | | |
| x17 | 2. 411 | 2. 411 | 2. 970 | 2. 747 | 2. 970 | 2. 411 | 2. 565 | 2. 411 | 2. 565 | 2. 054 | 1. 872 | 2. 565 | 2. 970 | 2. 970 | 2. 565 | 1. 718 | 4. 357 | | | | |
| x18 | 2. 159 | 2. 159 | 2. 411 | 2. 054 | 3. 664 | 2. 747 | 1. 959 | 2. 565 | 2. 747 | 2. 565 | 2. 054 | 2. 054 | 2. 565 | 2. 565 | 2. 411 | 2. 277 | 2. 747 | 2. 970 | | | |
| x19 | 2. 747 | 2. 970 | 1. 792 | 2. 159 | 2. 747 | 2. 411 | 2. 054 | 2. 747 | 1. 959 | 2. 159 | 2. 277 | 2. 565 | 2. 747 | 2. 159 | 2. 411 | 2. 747 | 2. 747 | 2. 747 | 3. 258 | | |
| x20 | 2. 277 | 2. 054 | 2. 054 | 2. 565 | 2. 747 | 2. 747 | 3. 258 | 2. 277 | 2. 970 | 2. 565 | 2. 565 | 2. 411 | 2. 970 | 2. 565 | 2. 411 | 2. 277 | 2. 411 | 2. 747 | 2. 411 | 3. 664 | |
| x21 | 2. 411 | 2. 054 | 1. 792 | 2. 747 | 2. 411 | 2. 747 | 2. 565 | 2. 747 | 2. 970 | 2. 277 | 2. 411 | 2. 970 | 2. 747 | 2. 747 | 2. 411 | 2. 565 | 2. 159 | 2. 970 | 2. 277 | 2. 565 | 3. 664 |

**Fig. 1** Matrix list of sequence scoring

| | x1 | x2 | x3 | x4 | x5 | x6 | x7 | x8 | x9 | x10 | x11 | x12 | x13 | x14 | x15 | x16 | x17 | x18 | x19 | x20 | x21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| x1 | 1 | | | | | | | | | | | | | | | | | | | | |
| x2 | 0. 867 | 1 | | | | | | | | | | | | | | | | | | | |
| x3 | 0. 934 | 0. 625 | 1 | | | | | | | | | | | | | | | | | | |
| x4 | 0. 864 | 0. 812 | 0. 925 | 1 | | | | | | | | | | | | | | | | | |
| x5 | 0. 74 | 0. 663 | 0. 912 | 0. 66 | 1 | | | | | | | | | | | | | | | | |
| x6 | 0. 864 | 0. 63 | 0. 727 | 0. 66 | 0. 24 | 1 | | | | | | | | | | | | | | | |
| x7 | 0. 658 | 0. 658 | 0. 75 | 0. 89 | 0. 59 | 0. 511 | 1 | | | | | | | | | | | | | | |
| x8 | 0. 829 | 0. 748 | 0. 748 | 0. 88 | 0. 88 | 0. 829 | 0. 93 | 1 | | | | | | | | | | | | | |
| x9 | 0. 575 | 0. 787 | 0. 912 | 0. 74 | 0. 7 | 0. 74 | 0. 7 | 0. 84 | 1 | | | | | | | | | | | | |
| x10 | 0. 787 | 0. 74 | 0. 74 | 0. 69 | 0. 53 | 0. 699 | 0. 63 | 0. 79 | 0. 74 | 1 | | | | | | | | | | | |
| x11 | 0. 812 | 0. 712 | 0. 776 | 1 | 0. 74 | 0. 684 | 0. 66 | 0. 92 | 0. 9 | 0. 95 | 1 | | | | | | | | | | |
| x12 | 0. 622 | 0. 75 | 0. 561 | 0. 75 | 0. 62 | 0. 7 | 0. 7 | 0. 75 | 0. 7 | 0. 56 | 0. 62 | 1 | | | | | | | | | |
| x13 | 0. 575 | 0. 63 | 0. 663 | 1 | 0. 91 | 0. 74 | 0. 7 | 1 | 0. 6 | 0. 66 | 0. 84 | 0. 6 | 1 | | | | | | | | |
| x14 | 0. 934 | 0. 748 | 0. 748 | 0. 71 | 0. 83 | 0. 934 | 0. 71 | 0. 71 | 0. 65 | 0. 93 | 0. 65 | 0. 93 | 0. 88 | 1 | | | | | | | |
| x15 | 0. 801 | 0. 888 | 0. 842 | 0. 89 | 0. 8 | 0. 699 | 0. 59 | 0. 67 | 0. 94 | 0. 89 | 0. 84 | 0. 89 | 0. 89 | 0. 8 | 1 | | | | | | |
| x16 | 0. 889 | 0. 7 | 0. 658 | 0. 66 | 0. 81 | 0. 75 | 0. 66 | 0. 81 | 0. 62 | 0. 62 | 0. 7 | 0. 81 | 0. 89 | 0. 75 | 0. 75 | 1 | | | | | |
| x17 | 0. 553 | 0. 553 | 0. 682 | 0. 63 | 0. 68 | 0. 553 | 0. 59 | 0. 55 | 0. 59 | 0. 47 | 0. 43 | 0. 59 | 0. 68 | 0. 68 | 0. 59 | 0. 39 | 1 | | | | |
| x18 | 0. 727 | 0. 727 | 0. 812 | 0. 69 | 1. 23 | 0. 925 | 0. 66 | 0. 86 | 0. 93 | 0. 86 | 0. 69 | 0. 69 | 0. 86 | 0. 86 | 0. 81 | 0. 77 | 0. 93 | 1 | | | |
| x19 | 0. 843 | 0. 912 | 0. 55 | 0. 66 | 0. 84 | 0. 74 | 0. 63 | 0. 84 | 0. 6 | 0. 66 | 0. 7 | 0. 79 | 0. 84 | 0. 66 | 0. 74 | 0. 84 | 0. 84 | 0. 84 | 1 | | |
| x20 | 0. 622 | 0. 561 | 0. 561 | 0. 7 | 0. 75 | 0. 75 | 0. 89 | 0. 62 | 0. 81 | 0. 7 | 0. 7 | 0. 66 | 0. 81 | 0. 7 | 0. 66 | 0. 62 | 0. 66 | 0. 75 | 0. 66 | 1 | |
| x21 | 0. 658 | 0. 561 | 0. 489 | 0. 75 | 0. 66 | 0. 75 | 0. 7 | 0. 75 | 0. 81 | 0. 62 | 0. 66 | 0. 81 | 0. 75 | 0. 75 | 0. 66 | 0. 7 | 0. 59 | 0. 81 | 0. 62 | 0. 7 | 1 |

**Fig. 2** Scores list of correlation between the sequences

Figure 3 showed that when alpha was 0.8, the classification results were very clear. {*X*2, X17} and {X18, X19} were clustered into one group respectively, which suggested that the relevance between sequences in each category (such as *X*2 and X17 in {*X*2, X17}) was much higher. The similar analysis could be done when α was 0.6, 0.4 and 0.2 respectively.

Through clustering analysis of fuzzy relation R, we could divide fuzzy relation R into two categories as shown in Fig. 3, one was {X17, *X*2, X4, X3, X18, X19, X12, X1, X5, X20, X7, X21, X6}, the other was {X16, X10, X15, X13, X11, X14, X8, X9}. Inferring from the fuzzy clustering analysis, there were some similarities in gene features and these genes were corresponded to element clustered in the same kind. For example, genes of the group {*EP300, JUN, MAPK3, MAFG, FOS, FOSB, Runx2, IL6, NFA5, GFAP, OS9, OSM, LBSP*}corresponding to the element in the group {X17, *X*2, X4, X3, X18, X19, X12, X1, X5, X20, X7, X21, X6} have similar function. The formation and reconstruction of bone

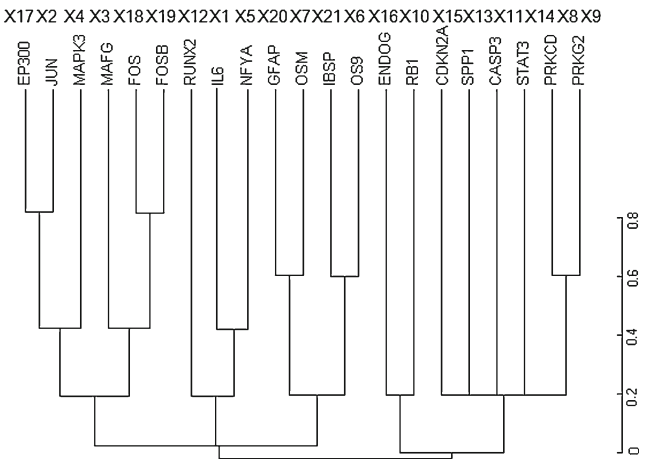


**Fig. 3** Division tree diagram of different threshold values. 0-0.8 on the right is the branch condition corresponding to α with different threshold values

mainly included the differentiation and proliferation of bone progenitor cells and cartilage progenitor cells and the formation of extracellular matrix, which was closely related to the activation and inhibition of a series of genes and osteoblast-specific transcription factor played an important role in regulation of these genes. Furthermore, *RUNX2* played an important role in the regulation of skeletal-related genes [10]. *RUNX2* has been identified as a high risk factor and directly related gene in osteosarcoma, but there were few studies on the relationship between the rest of the gene sequences in the same class and osteosarcoma, thereby we could further deduce the genes corresponding to {*X*2, X4, X17, X3, X18, X19, X12, X1, X5, X20, X7, X21, X6} have similar relationship as Runx2 with osteosarcoma, which may be understanded as these genes were all important risk factors and directly related genes in bone marrow.

## Discussion

As scientific research is becoming more and more deepen, its study objects are getting more and more complex too. Especially in the field of medical research, some certain complex phenomenons are difficult to be analyzed and solved well with precise mathematical methods before. Fuzzy clustering analysis is a new method to deal with the ambiguous classification, and also a powerful mathematical tool to describe and deal with fuzzy information. This method has been widely used in basic medical researches and clinical studies and good results are achieved because of its theoretical characteristics [18, 19]. Clustering methods can be roughly divided into two groups: hierarchical and partitional methods [20]. The results of hierarchical methods are represented as dendrograms, each branch representing a group of genes with similar behavior [21]. We selected hierarchical fuzzy clustering method and the genes

related to osteosarcoma were divided into two groups, the genes in {*EP300, JUN, MAPK3, MAFG, FOS, FOSB, Runx2, IL6, NFA5, GFAP, OS9, OSM, LBSP*}were speculated had similar function as Runx2, as the role of Runx2 was to attenuate osteoblast growth and promote bone phenotype maturation in osteogenic lineage, so did the rest genes [22–24]. In fact, many researches confirmed our speculation was right. For example, OncostatinM (OSM), a multifunctional cytokine belonging to the Interleukin (IL)-6 family, could induce chronic joint inflammation and destruction [25] and growth inhibition of various solid tumor cell lines derived from melanoma [26], breast and lung cancer [27] hepatoma [28] glioblastoma[29] or osteosarcoma [30]. OSM products resulted in localized joint inflammation, but also in the formation of several layers of osteoblastic-like cells and new bone formation [31], and evidence showed that short term of OSM treatment stimulated osteogenesis but long term of OSM treatment inhibited bone nodules formation [32]. FOS and FOSB were c-fos related genes, it was reported that c-Fos and related genes knock-out mice lack osteoclasts and developed the bone remodeling disease osteopetrosis [33] [34], demonstrating that c-Fos and its related genes were essential genes for osteoclast differentiation and bone remodeling in general [35].

Another gene interleukin-6(IL-6) which was a multifunctional cytokine involved in osteoclast recruitment and differentiation into mature osteoclasts [36, 37] Osteoblast-derived IL-6 was crucial to bone remodeling particularly [38]. All the reports mentioned above proved that the genes clustered into one group had analogous function and the fuzzy clustering method applied in this study was feasible and it was much easier and more convenient to analyze biologically relevant groups of genes.

Fuzzy clustering analysis of osteosarcoma-related genes was to integrate the information of the same problem related genes, so the relationship among them could be understood from a broader and deeper framework. And this was an important issue in genomics research.

The method used in this study differed from conventional methods utilized in the research of protein networks, function of gene products, which used a completly gene sequence-based approach, and extended from a perspective of similar sequence mechanism to sinilar function mechanism, not only meaningful for the research of myeloma disease, but also other related diseases. Medical-related studies were needed in order to obtain definitive conclusion of fuzzy clustering analysis and the results in this study just provided potential genetic refrences for the prevention and clinical diagnosis for osteosarcoma.

**Conflict of Interest** The authors declare that there are no any conflicts of interest.

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