European Review for Medical and Pharmacological Sciences 2016; 20: 2799-2804

NKD2 a novel marker to study the progression of osteosarcoma development

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Abstract. – OBJECTIVE: Osteosarcoma is the most frequent metastatic bone tumor in the current era. An effective novel marker is essential at the current scenario for the identification of the tumor and to treat them in the early stage of osteosarcoma.

PATIENTS AND METHODS: In the present study, patients having metastatic and non-metastatic osteosarcoma samples were collected from the 23 patients along with the control. Microarray analysis was performed on both samples. The results were validated using Western blot analysis.

RESULTS: Microarray analysis confirms the up-regulation of NKD2 gene in the progression of osteosarcoma development. The results were validated using western blot analysis. Microarray in accordance with Western blot analysis helps to validate the expression of NKD2 in the progression of osteosarcoma development.

CONCLUSIONS: In short, NKD2 is the key molecular marker to study the progression of osteosarcoma development, and it may be used for better prognosis of the disease in early stage.

Key Words
Osteosarcoma, Metastasis, NKD2, Western blot.

Introduction

Spreading of cancerous cells is a complex process commonly called as metastasis. Osteosarcoma is the most common aggressive form of bone tumor occurs in children and young adults, at the same time in adults with age group greater than 50 years[1]. It was reported that the following organs, namely distal femoral, femur and proximal tibial metaphyses, are the major sites of osteosarcoma[2]. Osteosarcoma takes place due to abnormal osteoid production and differentiation along with genomic instability. The progression of osteosarcoma is associated with numerous genes with diverse genomic alterations[3]. In earlier days, surgical resection therapy is practiced in osteosarcoma patient. It leads to poor prognosis and after that prominent chemotherapy was developed in the 1980s, which increases the survival rate of osteosarcoma patients from 50 to 80%[4,5]. But there is no significant progress in therapeutic upgrading as well as in prognosis improvement in patients suffering from osteosarcoma[6]. Especially the patients with a metastatic form of osteosarcoma are having only 10-30% longer survival rates[7] and other exhibits less response. To improve the patient outcome for survival, a new therapeutic target is necessary to put forward to investigate the anti-metastatic activity.

Besides of many reasons like deletion, translocation, amplification and other mutations that contribute to tumor development[8], epigenetic changes can also one reason for the development of many cancers including osteosarcoma[9-11]. Osteosarcoma metastasis prognosis is not well-known studied[12,13]. It is essential to identify those candidate genes beyond the osteosarcoma metastasis process, which should be utilized as a molecular marker for early detection of metastatic osteosarcoma.

Patients and Methods

Patients

Tissue samples were collected from the patients having a metastatic and non-metastatic form of osteosarcoma, through proper approval and consent letter from the patients. The complete information about the patients, including the age and sex, were shown in Table I. Immediately after the collection of samples, it was kept in the liquid nitrogen and stored at –80°C until RNA extraction.

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Table I. Complete information of sampling from osteosarcoma patients.

SL.No	Patient ID	Tumor site	Sample collection	Age of patients	Patients sex
Osteosarcoma samples from patients not having metastasis					
1.	ONM0002	Tibia	Biopsy	14	Female
2.	ONM0053	Femur	"	19	Female
3.	ONM0060	Distal femur	"	20	Male
4.	ONM0074	Distal femur	"	14	Female
5.	ONM0081	Femur	"	21	Female
6.	ONM0085	Tibia	"	22	Male
7.	ONM00113	Femur	"	21	Male
8.	ONM00118	Distal femur	"	22	Male
9.	ONM00120	Tibia	"	11	Female
10.	ONM00126	Distal femur	"	19	Male
11.	ONM00132	Femur	"	21	Male
12.	ONM00137	Tibia	"	22	Female
Osteosarcoma samples from patients having metastasis					
13.	OSM0019a	Distal femur	"	17	Female
14.	OSM0028a	Distal femur	"	14	Male
15.	OSM0030a	Distal femur	"	17	Male
16.	OSM0035a	Tibia	"	10	Male
17.	OSM0047a	Distal femur	"	22	Female
18.	OSM0048a	Femur	"	22	Female
19.	OSM0059a	Distal femur	"	16	Female
20.	OSM0062a	Distal femur	"	14	Male
21.	OSM0096a	Tibia	"	17	Male
22.	OSM00107a	Distal femur	"	15	Female
23.	OSM00148a	Distal femur	"	22	Female

RNA Extraction

Total RNA was extracted from samples along with control samples using TRIzol reagent method (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instruction. RNA concentrations and quality were determined by NanoDrop (ND-1000 spectrophotometer from Thermo Fisher, Montchanin, DE, USA), and the integrity of the same was analyzed by agarose gel electrophoresis. The isolated RNA was used for further experiments.

Microarray Analysis

The mRNA transcripts and its expression were studied by cDNA microarray. The GeneChip Human Genome U133 Plus 2.0 array (Affymetrix from Santa Clara, CA, USA) was used to analyze the expression profiling of mRNA from osteosarcoma with and without metastasis samples. Biotinylated cRNA was synthesized from Poly A tailed mRNA by using the 3' IVT Express kit (Affymetrix from Santa Clara, CA, USA) according to the manufacturer's instructions, and the complete protocol has been got from the article of Iwasaki et al[14]. After fragmentation, 12.5 μg of cRNA were hybridized to the GeneChip array for 16 h. It was washed with buffer and stained accordingly using the GeneChip Fluidics Station 450 (Affymetrix from Santa Clara, CA, USA) and, then, the results were scanned using GeneChip Scanner 3000 (Affymetrix from Santa Clara, CA, USA). The complete protocol for the microarray has been got from the article of Iwasaki et al[14].

Western Blot Analysis

The cell lysate was prepared from the normal tissue as well as the metastatic and non-metastatic form of osteosarcoma tissues. The proteins are isolated and resolved in 10% SDS-PAGE gel. They are then transferred to the membrane and incubated with primary antibody (anti-Nkd2 antibody from Sigma-Aldrich, St. Louis, MO, USA) for overnight at 4°C. For loading controls, anti-β-actin and anti-transferrin antibodies from Abcam). The nonspecific binding of primary antibody is washed out and further incubated with the suitable secondary antibody (anti-β-actin and anti-transferrin antibodies from Abcam, Cambridge, UK). Later the signals are visualized with DAB kit.

Statistical Analysis

Basic statistical analysis such as average, mean and percentage were calculated using Microsoft Office Excel 2007 (Microsoft Corp., Redmond, WA, USA). Statistics were applied in tumor samples analysis by counting the tumor cells.'

Table II. Important transcripts expressed in osteosarcoma samples from patients affected by metastasis.

SL.	No
1.	NKD2
2.	TGF-β
3.	Osteopontin
4.	MAPK
5.	Runx2
6.	CLIC 5
7.	P53
8.	β 4 integrin
9.	Ezrin
10.	NSE2
11.	Smad
12.	RANKL
13.	IL-8

Results

A Sample Collected from the Patients with Osteosarcoma

Osteosarcoma is the most common cancer, at present accurate prognosis of metastasis stage of osteosarcoma is the essential need for effective treatment of the disease before spreading. Molecular markers are not characterized well for the prognosis of metastasis stage of osteosarcoma. Identification of osteosarcoma metastasis genes will provide a way to design new markers for the initial identification of metastasis during the early stage of osteosarcoma. Keeping with this in mind, the research work was planned to enumerate the genes responsible for metastasis of osteosarcoma. A biopsy was performed and the tissue samples were collected from the patients, which was summarized in the Table I. All the tumor samples were analyzed and confirmed to have >90% tumor cells, by a histological pathologist.

Microarray Analysis

To identify the molecular markers responsible for metastasis of osteosarcoma, the samples from the patients having the osteosarcoma at the stage of metastasis and without metastasis groups were subjected to RNA extraction followed by microarray. Samples were hybridized to the GeneChip array for 16 h, respectively. Many upregulations and downregulation of transcripts were noted, the one which is upregulated was listed in Table II along with its function. Also, based on the significance of preliminary microarray data (Figure 1) and interest, NKD2 gene was chosen and focused for further studies to confirm it as a molecular marker for detection of a metastatic form of osteosarcoma in initial stages.

Western Blot Analysis

To confirm the results and to validate the microarray data, a Western blot was performed. Based upon the interest and significance of preliminary microarray data (Figure 1), NKD2 gene was focused for further studies to confirm it as a molecular marker for detection of a metastatic form of osteosarcoma in initial stages. The tissue samples were subjected to Western blot with anti-NKD2 (from Sigma-Aldrich, St. Louis, MO, USA) and anti-β actin (from Cambridge, UK) antibodies. Similar experiments were performed from control tissues. The data of Western blotting were shown in Figure 2, which illustrates that the expression of NKD2 was observed in the samples from patients having metastatic osteosarcoma. Rather, NKD2 show restricted expression in the samples from patients having without metastatic osteosarcoma. Similar results were observed for all the samples.

To check the expression profile of NKD2 in the blood samples of patients with and without metastatic osteosarcoma, protein lysate was pre-

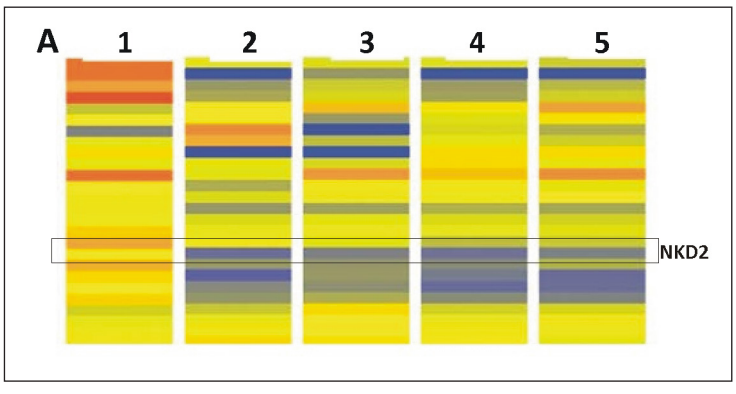

Figure 1. Microarray analysis. The osteosarcoma with metastasis samples was hybridized with the human gene chip. The upregulated expression of NKD2 transcripts was shown in the box. Lane 1 – Control, rest all are samples from osteosarcoma with metastasis.

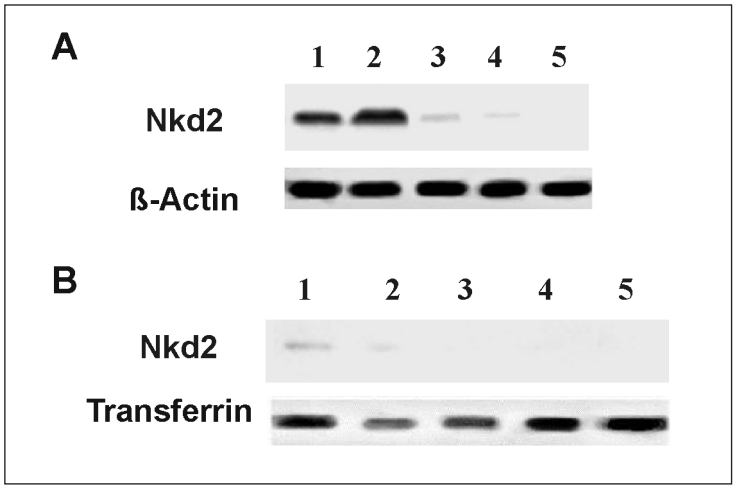

Figure 2. Western blot analysis. A. NKD2 and β-actin expression of osteosarcoma patient samples (with and without metastasis) along with control. Osteosarcoma with metastasis samples were loaded in lane 1-2; whereas lane 3-4 were loaded with osteosarcoma without metastasis patient's samples; lane 5 is the control. B. Similarly, Western blot analysis was performed on serum samples of osteosarcoma along with the control. Lane 1-2 – Osteosarcoma serum samples from patients having metastasis; Lane 3-4 indicates osteosarcoma serum samples from patients without metastasis; lane 5 is the control.

pared from serum samples followed by Western blot analysis with the control samples. The data were shown in Figure 3; they illustrate that the limited expression of NKD2 was identified in serum samples of metastatic osteosarcoma patients. Rather, NKD2 were not observed in the serum samples of other groups and the control samples.

Discussion

The mouse model with osteosarcoma has the ability to resemble histologically with human cancer, and that greatly helps to understand their disease biology. Using a mouse model for human disease is not good for studying certain kinds of diseases, as the osteosarcoma. A range of new developments was enumerated for the patients struggling with osteosarcoma, but the detection of the osteosarcoma and its metastasis in early stages is not well-known understand[12-13]. For characterization and identification of the patients having the osteosarcoma at the initial stage of metastasis for the successful treatment, it is essential to identify the genes behind osteosarcoma metastasis. In the present study, Microarray along with Western blot was used to identify the patients affected by the osteosarcoma at the stage of metastasis and its responsible genes. These research findings highlight the essential milestones on the identification of osteosarcoma metastasis genes.

The data of microarray show that expression of following transcripts in the samples of patients having the osteosarcoma at the stage of metastasis, which was tabulated in Table II. In order to confirm the preliminary data of microarray, Western blot was performed and the data were shown in Figure 2A. The data confirm that the NKD2 is specific of osteosarcoma, expressed during the stage of metastasis.

To continue the study, blood samples were collected from osteosarcoma with and without metastasis patients along with controls and serum were prepared followed by immunoblot analysis. The data in Figure 2B shows that the detection of NKD2 was observed in the samples of patients having the osteosarcoma at the stage of metastasis, which confirms that it is specific to osteosarcoma, expressed during the stage of metastasis. In addition, partial or restricted expression of NKD2 was noted in a patient sample containing osteosarcoma without having the stage of metastasis. The reason may be due the patients are looking near forward to metastasis stage of osteosarcoma. The data suggest that the presence of NKD2 could be employed for prognosis of osteosarcoma at the stage of metastasis.

Many proteins have been expressed during osteosarcoma metastasis, as Ezrin, which is a membrane-binding N-terminal FERM domain[15-16] that has an important function not only in osteosarcoma metastasis, but also in the pancreatic and mammary adenocarcinomas[17-19]. Notably in the current study the expression of NKD2 was noted in the serum samples of osteosarcoma with metastasis patients and restricted expression in osteosarcoma without metastasis patients illustrates that it could be employed as a molecular marker. Further studies are important to develop NKD2 as a new molecular marker to detect the metastasis of osteosarcoma in initial stages.

Similarly, integrins are heterodimers consist of α and β subunits, which regulates metastasis, cell adhesion, survival, angiogenesis, oncogene-

sis, signaling, migration and proliferation[20-24]. In addition to the reports, it was also identified that implication of β4 integrin is involved in various cancer progression[25-29]. Of note, our data from Figures 2 and 3 confirm that the NKD2 expression in patients sample affected by the osteosarcoma at the stage of metastasis, which mean that NKD2 is a transcript responsible specifically for osteosarcoma metastasis. Conversely, restricted expression of NKD2 was detected in the patient samples of osteosarcoma but not at the stage of metastasis. The reason may be due to the patients who are looking near forward to the metastatic stage of osteosarcoma. With this importance, it could be engaged as a molecular marker. Additional characterization is important to generate NKD2 as a new marker for the identification of the osteosarcoma at the early stage of metastasis.

Conclusions

The present study concludes that the transcripts namely NKD2 were expressed strongly in patients having the osteosarcoma at the stage of metastasis. In accordance with the microarray experiments along with Western blotting, it was validated and confirmed that the NKD2 is specific to osteosarcoma, expressed during the stage of metastasis. Also, to expand NKD2 as a novel marker, further characterization is important for the identification of the osteosarcoma metastasis in early stages.

Acknowledgements:
The authors were thankful to patients and grateful for the institutional support for the scuccesfully completing the project.

Declaration of Conflict of Interest:
The authors declare no conflict of interest in any part of the manuscript.

References

1) Picci P. Osteosarcoma (osteogenic sarcoma). Orphanet J Rare Dis 2007; 2: 6..
2) Ottaviani G, Jaffe N. The epidemiology of osteosarcoma. Cancer Treat Res 2009; 152: 3-13.
3) Smida J, Baumhoer D, Rosemann M, Walch A, Bielack S, Poremba C, Remberger K, Korsching E, Scheurlen W, Dierkes C. Genomic alterations and allelic imbalances are strong prognostic predictors in osteosarcoma. Clin Cancer Res 2010; 16: 4256-4267.
4) Ottaviani G, Jaffe N. The epidemiology of osteosarcoma. Pediatric and Adolescent Osteosarcoma: Springer, 2010; pp. 3-13.
5) Tan ML, Choong PF, Dass CR. Osteosarcoma–conventional treatment vs. gene therapy. Cancer Biol Ther 2009; 8: 106-117.
6) Allison DC, Carney SC, Ahlmann ER, Hendifar A, Chawla S, Fedenko A, Angeles C, Menendez LR. A meta-analysis of osteosarcoma outcomes in the modern medical era. Sarcoma 2012; 2012: 704872.
7) Meyers PA. Muramyl tripeptide (mifamurtide) for the treatment of osteosarcoma. Expert Rev Anticancer Ther 2009; 9: 1035-1049.
8) Szuhai K, Cleton-Jansen A-M, Hogendoorn PC, Bovée JV. Molecular pathology and its diagnostic use in bone tumors. Cancer Genet 2012; 205: 193-204.
9) Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. Nat Rev Genet 2007; 8: 286-298.
10) Das PM, Ramachandran K, VanWert J, Ferdinand L, Gopisetty G, Reis IM, Singal R. Methylation mediated silencing of TMS1/ASC gene in prostate cancer. Mol Cancer 2006; 5: 28.
11) Al-Romaih K, Sadikovic B, Yoshimoto M, Wang Y, Zielenska M, Squire JA. Decitabine-induced demethylation of 5′ CpG island in GADD45A leads to apoptosis in osteosarcoma cells. Neoplasia 2008; 10: 471-480.
12) Meyers PA, Heller G, Healey JH, Huvos A, Applewhite A, Sun M, LaQuaglia M. Osteogenic sarcoma with clinically detectable metastasis at initial presentation. J Clin Oncol 1993; 11: 449-453.
13) Harris MB, Gieser P, Goorin AM, Ayala A, ShochatSJ, Ferguson WS, Holbrook T, Link MP. Treatment of metastatic osteosarcoma at diagnosis: a Pediatric Oncology Group Study. J Clin Oncol 1998; 16: 3641-3648.
14) Iwasaki T, Tanaka K, Kawano M, Itonaga I, Tsumura H. Tumor-suppressive microRNA-let-7a inhibits cell proliferation via targeting of E2F2 in osteosarcoma cells. Int J Oncol 2015; 46: 1543-1550.
15) LouvetÐVallée S. ERM proteins: from cellular architecture to cell signaling. Biol Cell 2000; 92: 305-316.
16) Bretscher A, Edwards K, Fehon RG. ERM proteins and merlin: integrators at the cell cortex. Nat Rev Mol Cell Biol 2002; 3: 586-599.
17) Khanna C, Khan J, Nguyen P, Prehn J, Caylor J, Yeung C, Trepel J, Meltzer P, Helman L. Metastasis-associated differences in gene expression in a murine model of osteosarcoma. Cancer Res 2001; 61: 3750-3759.
18) Khanna C, Wan X, Bose S, Cassaday R, Olomu O, Mendoza A, Yeung C, Gorlick R, Hewitt SM, Helman LJ. The membrane-cytoskeleton linker ezrin is necessary for osteosarcoma metastasis. Nat Med 2004; 10: 182-186.
19) Nestl A, Von Stein OD, Zatloukal K, Thies W-G, Herrlich P, Hofmann M, Sleeman JP. Gene expression

patterns associated with the metastatic phenotype in rodent and human tumors. Cancer Res 2001; 61: 1569-1577.

20) Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell 2002; 110: 673-687.

21) Howe A, Aplin AE, Alahari SK, Juliano R. Integrin signaling and cell growth control. Curr Opin Cell Biol 1998; 10: 220-231.

22) Hood JD, Cheresh DA. Role of integrins in cell invasion and migration. Nat Rev Cancer 2002; 2: 91-100.

23) Aplin AE, Howe AK, Juliano R. Cell adhesion molecules, signal transduction and cell growth. Curr Opin Cell Biol 1999; 11: 737-744.

24) Giancotti FG, Ruoslahti E. Integrin signaling. Science 1999; 285: 1028-1033.

25) Mariotti A, Kedeshian PA, Dans M, Curatola AM, Gagnoux-Palacios L, Giancotti FG. EGF-R signaling through Fyn kinase disrupts the function of integrin α6β4 at hemidesmosomes role in epithelial cell migration and carcinoma invasion. J Cell Biol 2001; 155: 447-458.

26) Nikolopoulos SN, Blaikie P, Yoshioka T, Guo W, Giancotti FG. Integrin β4 signaling promotes tumor angiogenesis. Cancer Cell 2004; 6: 471-483.

27) Mercurio AM, Rabinovitz I. Towards a mechanistic understanding of tumor invasion – lessons from the α6 β4 integrin. Seminars in cancer biology: Elsevier, 2001; pp. 129-141.

28) Guo W, Pylayeva Y, Pepe A, Yoshioka T, Muller WJ, Inghirami G, Giancotti FG. β4 integrin amplifies ErbB2 signaling to promote mammary tumorigenesis. Cell 2006; 126: 489-502.

29) Trusolino L, Bertotti A, Comoglio PM. A Signaling adapter function for α6β4 integrin in the control of HGF-dependent invasive growth. Cell 2001; 107: 643-654.