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Original Article

Expression and clinical significance of Dickkofp-1 (DKK-1) in neuroblastoma

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Abstract: Objective: Our aims were to investigate the expression and clinical significance of Dickkopf-1 (DKK-1) in human neuroblastoma (NB). Methods: We examined the expression of DKK-1 by immunohistochemical staining (IHC) in 42 human NB tissues, 19 human ganglioneuroma tissues (GN) and 15 normal human adrenal gland tissues. We then determined the relative abundance of DKK-1 mRNA and protein in three NB cell lines (SH-SY5Y, SK-N-SH and IMR-32) using qRT-PCR and Western blotting. Results: DKK-1 was expressed in normal human adrenal tissues, and its level was significantly reduced in human GN tissues and markedly reduced in NB tissues ($P<0.001$). Moreover, DKK-1 protein and mRNA were profoundly decreased in NB cell lines compared to control HEK293 cells ($P<0.001$). Conclusion: DKK-1 was down-regulated in human NB tissues and NB cell lines. These observations suggested that DKK-1 could be a potential molecular biomarker for NB risk stratification and prognosis estimation in patients.

Keywords: Neuroblastoma (NB), Ganglioneuroma (GN), DKK-1, gene expression diagnosis, prognosis

Introduction

Neuroblastoma (NB) is one of the most common malignant solid tumors in children. It is the third most common malignant neoplasm of childhood, after leukemia and central nerve system (CNS) tumors. The incidence of pediatric NB is 0.3-5.5/per 100,000 person-years, and 80% of cases occur in young children under 5 years old [1, 2]. It represents only 8-10% of all cases of childhood malignancy, yet results in 15% of cancer death in children [3]. Despite multimodal treatment, survival rates for high-risk NB patients remain disappointingly low [4] and the overall 5-year survival of NB patients is only 59% [5, 6]. Thus, there is an urgent need to identify specific molecular biomarkers for early NB diagnosis, risk stratification and therapeutic efficacy monitor.

NB is originated from neural crest and its pathogenesis is largely unknown [3]. Dickkopf-1 (DKK-1) is a newly identified secretory glycoprotein that belongs to the evolutionally conserved DKK family [7]. As one of the potent antagonist of Wnt signal cascades, DKK-1 has been increasingly implicated in the cancer development, while its role in the pathogenesis of NB is not completely understood [3]. This study aimed to investigate the expression and clinical significance of DKK-1 in human NB.

Methods

Study population

All human study protocols were approved by the Institutional Review Board of the First Affiliated Hospital of Zhengzhou University, Henan Province, China. All study participants' guardians provided written informed consent. We collected surgical resection of suspected tumor tissues from the patients who underwent surgery in Department of Pediatric Surgery at the First Affiliated Hospital to Henan University between Jan, 2010 and May, 2015. 42 samples of NB were collected from 22 male and 20 female subjects, ranging in age from 3 months to 6 years (average age 89 ± 38 months); 19 samples of ganglioneuroma (GN) were collected from 11 male and 8 female subjects, ranging in age from 5 months to 5 years (average age 76 ± 41 months). None of the subjects had chemotherapy or radiotherapy before surgery.

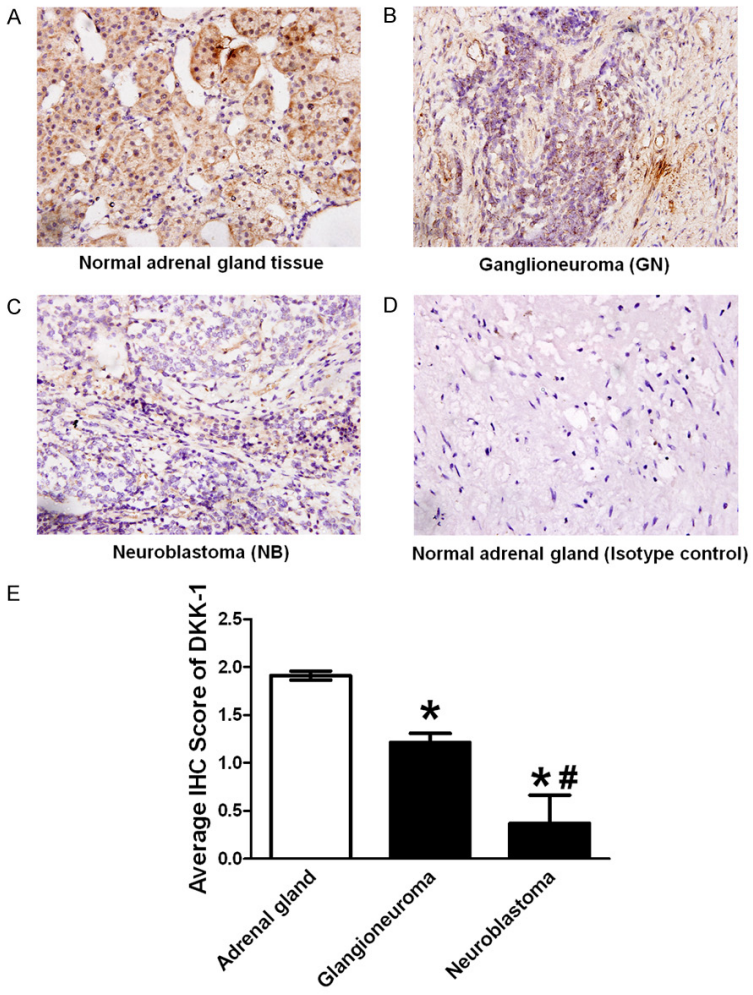

Figure 1. Expression of DKK-1 in human NB, ganglioneuroma (GN) and normal adrenal gland tissues. Representative immunohistochemical (IHC) staining of DKK-1 with positive signals in brown: (A) normal adrenal gland, (B) ganglioneuroma (GN), (C) neuroblastoma (NB), (D) normal adrenal gland stained with isotype IgG control. (E) Average IHC scores of DKK-1 expression in normal adrenal gland (n = 15), NB (n = 42) and GN (n = 19). *P<0.001 compared with normal controls, #P<0.001 compared with IHC score of GN.

15 normal adrenal glands tissues were obtained from age-matched subjects from the Department of Pathology at our hospital. Freshly collected tissues were stored in -70°C freezers prior to being serially sectioned with 5 μm in thickness.

Cell cultures

Human NB cell lines IMR-32, SK-N-SH and SH-SY5Y were purchased from China Centre for Type Culture Collections (CCTCC), and HEK293 controls were the stocks in the laboratory. Monolayer cells were culture in a humidified incubator at 37°C with 5% CO_2. All the culture media were supplied with 10% FBS. IMR-32 cells were cultured in EMEM medium; SK-N-SH cells and HEK293 were cultured in DMEM medium; Sh-SY5Y cells were cultured in RPMI-1640 medium.

Immunohistochemical staining (IHC)

IHC was performed on frozen-sections. Briefly, sections were fixed with acetone at 4°C for 10 min, and were then incubated with 30 mmol/L H_2O_2 for 20 mins. Sections were then incubated with polyclonal rabbit anti-human DKK-1 antibody (diluted 1:100, Epitomics) at 4°C overnight. Signals were amplified by subsequent incubation with an avidin-biotin complex (SP9001, Beijing Zhong Shan-Golden Bridge Biological Technology CO., LTD) and DAB chromatogenic staining (ZLI-9032, Beijing Zhong Shan-Golden Bridge Biological Technology CO., LTD). Isotype control IgG was used as the negative control for each sample. Images were acquired by Olympus DD70 BX51 system with 400 × magnification, and cells with brown staining in both the cytoplasma and plasma membrane were judged as positive. An average score of 10 randomly picked fields from each sample was calculated. More than 100 cells were analyzed in each field and scored by the percentage of DKK-1 positive cells in each field: 0-20%, 0; 20-50%, 1; >50%, 2.

Quantitative real-time PCR (qRT-PCR)

Total RNA from each groups of cell was isolated using TRIzol reagent (TaKaRa, Dalian) following the manufacturer's protocol, and reverse transcribed to cDNA (TaKaRa RT Kit). The expression of DKK-1 transcript was determined by real-time PCR using SYBR Premix Ex Taq (TaKaRa Biotechnology Co. Ltd, Dalian, China)

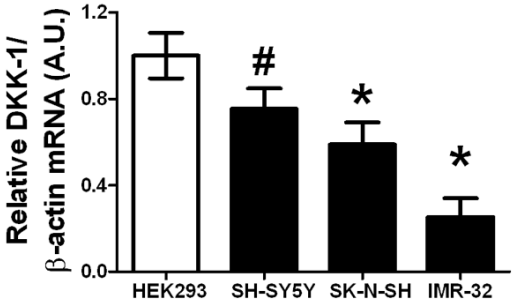

Figure 2. Relative *DKK-1* mRNA in cultured NB cell lines. Shown are relative *DKK-1* mRNA to β-ACTIN in NB cell lines (SH-SY5Y, SK-N-SH and IMR-32) and control cells HEK293. *P<0.001 compared with HEK293; #P<0.001 compared with HEK293; n = 3 separated experiments in triplicates.

and iCycler iQ™ Real-Time PCR Detection System (BIO-RAD). Forward primer: 5'-ACCTCCATAGAAGATTCTAGAGCCACCATGATGGCTCTGGGC-3'; reverse primer: 5'-GCGATCGCAGATCCTTGCGGCCGCTTAGTGTCTCTGACA-3' for DKK-1. Forward primer: 5'-AAGGCCAACCGCGAGAA-3' and reverse primer: 5'-CCTCGTAGATGGGCACA-3' for *β-ACTIN* as controls. All primers were designed and synthesized by TaKaRa Biotechnology Co., Ltd (Dalian, China). The reaction mixture (30 μL) was preheated at 94°C for 5 min, followed by 30 amplification cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s and extension at 68°C for 1 min, and ended at 68°C for 5 min. Each sample was analyzed in three separate experiments in triplicates. The fold-change in gene expression relative to *β-ACTIN* was calculated by $2^{-\Delta\Delta}$CT.

Western blotting analysis

Total protein was extracted from cultured monolayers (Epitomics) and 60 μg proteins per lane were separated by SDS-PAGE. After electrophoresis, proteins were transferred to the PVDF membranes. The membranes were blocked and then incubated with polyclonal rabbit anti-human DKK-1 antibody (1:1000) followed by HRP-conjugated goat anti-rabbit antibody (1:1000). Labeled bands were detected using the ECL chemiluminescent kit (Sigma). β-actin was used as loading control. The levels of DKK-1 and β-actin in 4 cell lines were quantified using Image J and analyzed from three separate experiments.

Statistics

The SPSS was used to perform all statistical analysis. Data were presented as mean ± SEM. Group means of parametric variables were compared using ANOVA followed by post-hoc Bonferroni's multiple comparison analyses. Comparisons of non-parametric variables were performed using Kruskal-Wallis Test followed by Dunn's multiple comparisons analysis. A 2-tailed value of $P<0.05$ was considered as statistically significant.

Results

Differential expression of DKK-1 in normal adrenal gland, GN and NB tissues

In the normal adrenal gland tissues, DKK-1 was highly expressed and mainly located in cytoplasm and on plasma membrane (**Figure 1A**, IHC score = 1.912 ± 0.047). DKK-1 signal was barely detectable in adrenal gland tissues stained with IgG isotype control antibodies as the negative control (**Figure 1D**). In contrast, DKK-1 expression was markedly reduced in NB tissues (**Figure 1C**, IHC score = 0.371 ± 0.293), and moderately decreased in GN tissues (**Figure 1B**, IHC score = 1.214 ± 0.095). Quantification data revealed that the DKK-1 levels were significantly different among the different tissues (ANOVA, F = 211.375, P<0.001), and post-hoc analyses indicated that the DKK-1 protein was significantly reduced in GN and NB compared to normal adrenal gland (Bonferroni, P<0.01 vs. control).

DKK-1 mRNA in NB cell lines

Relative mRNA of DKK-1 were measured by qRT-PCR in the control HEK293 cells and in three NB cell lines with different differentiation levels: IMR-32, SK-N-SH and SH-SY5Y. As shown in **Figure 2**, the relative DKK-1 mRNA over housekeeping gene β-actin in IMR-32 (0.253 ± 0.087), SK-N-SH (0.589 ± 0.102), SH-SY5Y (0.754 ± 0.938) and control HEK293 cells (1.000 ± 0.106) were significantly different (ANOVA, $F = 57.628$, P<0.001). All three NB cell lines had significantly reduced DKK-1 mRNA compared with control HEK293 cells (SH-SY5Y *vs.* control: P<0.05; SK-N-SH *vs.* control, IMR-32 *vs.* control, P<0.001).

DKK-1 protein in NB cells lines

In line with the mRNA analysis, Western blot analysis revealed that the DKK-1 protein was differentially expressed in NB cell lines (ANOVA, $F = 156.783$, P<0.001). The relative level of

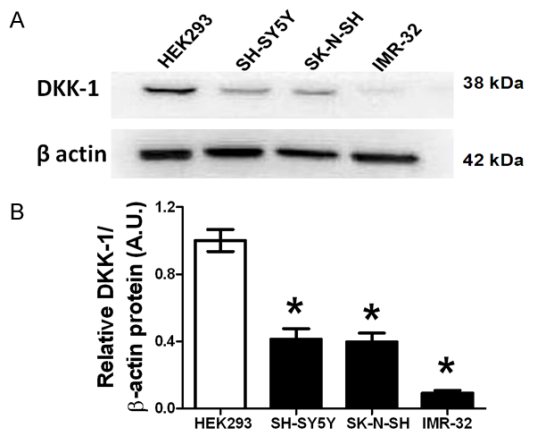

Figure 3. DKK-1 expression in cultured NB cell lines. A. Representative immunoblots showing the decreased expression of DKK-1 in three NB cell lines compared with control HEK293 cells. B. Densitometric quantification of DKK-1 to β-actin ratios was shown. *P<0.001 compared with HEK293, n = 3 separated experiments in triplicates.

DKK-1 protein to β-actin was significantly reduced in IMR-32 (0.093 ± 0.016), SK-N-SH (0.397 ± 0.051) and SH-SY5Y (0.411 ± 0.062) cell lines normalized to DKK-1 in HEK293 cells (1.000 ± 0.066) (**Figure 3**, all P<0.001). Interestingly, we found that the least differentiated cell line IMR-32 expressed the lowest level of DKK-1 mRNA (**Figure 2**) and proteins (**Figure 3**) among all the tested NB cell lines, suggesting the expression of DKK-1 was positively associated with cell differentiation.

Discussion

Neuroblastoma is one of the most common pediatric solid tumors originating from the sympathoadrenal lineage of neural crest [8]. The tumors are often occult in onset and have highly unpredictable malignant behaviors. Thus, there has been increasing demand to find novel NB specific molecular marker to provide early NB diagnosis, estimate the prognosis, develop personalized therapeutics and monitor treatment efficacy.

DKK-1 was originally discovered as a secreted antagonist for Wnt signaling during Xenopus embryogenesis, which is required for Xenopus head development [7]. Recently, DKK-1 has been shown to inhibit Wnt signaling in various types of cells in vertebrates [9], while its expression and functions in human tumors remain controversial. DKK-1 is highly expressed human non-small cell lung cancer (NSCLC) tissues, as well as NSCLC cell lines. Its level is positively associated with lymph nodes metastasis, and is inversely associated with 5-year disease free survival (DFS) rate in patients [10, 11]. DKK-1 is also highly expressed in human hepatocellular carcinoma, intrahepatic cholangiocarcinoma, esophageal carcinoma and prostate cancers [12-16]. Evidences from animal models and cultured cells have suggested that DKK-1 can enhance tumor cell migration and metastasis. Interestingly, DKK-1 expression is low in colon cancers, thyroid carcinoma, renal carcinoma and endometrial cancer [17-21], and upregulation of DKK-1 prevented the abovementioned cancer cell proliferation and migration.

The expression and the roles of DKK-1 in NB are not fully understood, and several related studies have yield controversial results. Wang *et al.* reported that DKK-1 induced apoptosis in the NB cell line SH-SY5Y and inhibited cell migration [22]. Koppen *et al.* also found that the amplification of MYCN oncogene downregulated DKK-1 expression in NB tissues. Inhibition of DKK-1 by MYCN could stimulate cancer cell proliferation, suggesting the growth suppressive effect of DKK-1 on tumors [23]. Contrary to these findings, Grachi *et al.* observed that DKK-1 released from NB played a crucial role in blocking osteoprogenitor cells differentiation and promoting metastasis to the skeleton [24]. However, the expressions of DKK-1 in NB tissues were not determined in these two studies. Grachi *et al.* measured the level of secreted DKK-1 in the plasma from 92 NB patients and 57 control subjects, and found no difference between two groups [25]. Lou *et al.* showed that DKK-1 levels in cerebrospinal fluid (CSF-DKK1) from NB patients were significantly elevated compared with control subjects, and its levels was significantly decreased after effective chemotherapy [26]. Taken together, the expression of DKK1 in human NB tissues has never been definitively studied, and the underlying mechanisms require further investigation.

In this study, we utilized immunohistochemical staining to detect DKK-1 expression in human NB and GN tissues. For the first time, we observed that DKK-1 was markedly reduced in human NB tissues compared with normal adrenal gland. The level of DKK-1 in GN was significantly higher than in NB tissues but significant-

ly lower than that in normal adrenal glands. In line, we showed that the mRNA and protein of DKK-1 were down-regulated in three NB cell lines. The reduced DKK-1 mRNA and protein was closely association with low level of NB cell differentiation. These findings implied a potential role of DKK-1 in NB tumor cell differentiation. Thus, DKK-1 expression in NB tissues may serve as a novel biomarker for NB risk stratification and prognosis estimation in patients.

In summary, we demonstrated that the expression of DKK-1 was profoundly suppressed in human NB, but was mildly reduced in human GN compared to normal adrenal gland tissues. These findings suggested that DKK-1 could be a potential molecular biomarker for the differential diagnosis of NB, NB risk stratification and prognosis estimation in patients. Further studies to explore the function of DKK-1 in NB pathogenesis and to elucidate the underlying molecular mechanisms are required to improve the treatment of NB in patients.

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Disclosure of conflict of interest

None.

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References

[1] Izbicki T, Mazur J and Izbicka E. Epidemiology and etiology of neuroblastoma: an overview. Anticancer Res 2003; 23: 755-760.

[2] Maris JM, Hogarty MD, Bagatell R and Cohn SL. Neuroblastoma. Lancet 2007; 369: 2106-2120.

[3] David R, Lamki N, Fan S, Singleton EB, Eftekhari F, Shirkhoda A, Kumar R and Madewell JE. The many faces of neuroblastoma. Radiographics 1989; 9: 859-882.

[4] Yanez Y, Grau E, Rodriguez-Cortez VC, Hervas D, Vidal E, Noguera R, Hernandez M, Segura V, Canete A, Conesa A, Font de Mora J and Castel V. Two independent epigenetic biomarkers predict survival in neuroblastoma. Clin Epigenetics 2015; 7: 16.

[5] Maris JM. Recent advances in neuroblastoma. N Engl J Med 2010; 362: 2202-2211.

[6] Spix C, Pastore G, Sankila R, Stiller CA and Steliarova-Foucher E. Neuroblastoma incidence and survival in European children (1978-1997): report from the Automated Childhood Cancer Information System project. Eur J Cancer 2006; 42: 2081-2091.

[7] Glinka A, Wu W, Delius H, Monaghan AP, Blumenstock C and Niehrs C. Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. Nature 1998; 391: 357-362.

[8] Nakagawara A. Neural crest development and neuroblastoma: the genetic and biological link. Prog Brain Res 2004; 146: 233-242.

[9] Fedi P, Bafico A, Nieto Soria A, Burgess WH, Miki T, Bottaro DP, Kraus MH and Aaronson SA. Isolation and biochemical characterization of the human Dkk-1 homologue, a novel inhibitor of mammalian Wnt signaling. J Biol Chem 1999; 274: 19465-19472.

[10] Li S, Qin X, Guo X, Cui A, He Y, Wei S, Wang X and Shan B. Dickkopf-1 is oncogenic and involved in invasive growth in non small cell lung cancer. PLoS One 2013; 8: e84944.

[11] Kim IG, Kim SY, Kim HA, Kim JY, Lee JH, Choi SI, Han JR, Kim KC and Cho EW. Disturbance of DKK1 level is partly involved in survival of lung cancer cells via regulation of ROMO1 and gamma-radiation sensitivity. Biochem Biophys Res Commun 2014; 443: 49-55.

[12] Chen L, Li M, Li Q, Wang CJ and Xie SQ. DKK1 promotes hepatocellular carcinoma cell migration and invasion through beta-catenin/MMP7 signaling pathway. Mol Cancer 2013; 12: 157.

[13] Zhang H, Yu C, Dai J, Keller JM, Hua A, Sottnik JL, Shelley G, Hall CL, Park SI, Yao Z, Zhang J, McCauley LK and Keller ET. Parathyroid hormone-related protein inhibits DKK1 expression through c-Jun-mediated inhibition of beta-catenin activation of the DKK1 promoter in prostate cancer. Oncogene 2014; 33: 2464-2477.

[14] Tao YM, Liu Z and Liu HL. Dickkopf-1 (DKK1) promotes invasion and metastasis of hepatocellular carcinoma. Dig Liver Dis 2013; 45: 251-257.

[15] Tung EK and Ng IO. Significance of serum DKK1 as a diagnostic biomarker in hepatocellular carcinoma. Future Oncol 2012; 8: 1525-1528.

[16] Shi RY, Yang XR, Shen QJ, Yang LX, Xu Y, Qiu SJ, Sun YF, Zhang X, Wang Z, Zhu K, Qin WX, Tang ZY, Fan J and Zhou J. High expression of Dickkopf-related protein 1 is related to lymphatic metastasis and indicates poor progno-

sis in intrahepatic cholangiocarcinoma patients after surgery. Cancer 2013; 119: 993-1003.
[17] Saupe F, Schwenzer A, Jia Y, Gasser I, Spenle C, Langlois B, Kammerer M, Lefebvre O, Hlushchuk R, Rupp T, Marko M, van der Heyden M, Cremel G, Arnold C, Klein A, Simon-Assmann P, Djonov V, Neuville-Mechine A, Esposito I, Slotta-Huspenina J, Janssen KP, de Wever O, Christofori G, Hussenet T and Orend G. Tenascin-C downregulates wnt inhibitor dickkopf-1, promoting tumorigenesis in a neuroendocrine tumor model. Cell Rep 2013; 5: 482-492.
[18] Cho SW, Lee EJ, Kim H, Kim SH, Ahn HY, Kim YA, Yi KH, Park do J, Shin CS, Ahn SH, Cho BY and Park YJ. Dickkopf-1 inhibits thyroid cancer cell survival and migration through regulation of beta-catenin/E-cadherin signaling. Mol Cell Endocrinol 2013; 366: 90-98.
[19] Qi L, Sun B, Liu Z, Li H, Gao J and Leng X. Dickkopf-1 inhibits epithelial-mesenchymal transition of colon cancer cells and contributes to colon cancer suppression. Cancer Sci 2012; 103: 828-835.
[20] Yi N, Liao QP, Li ZH, Xie BJ, Hu YH, Yi W and Liu M. RNA interference-mediated targeting of DKK1 gene expression in Ishikawa endometrial carcinoma cells causes increased tumor cell invasion and migration. Oncol Lett 2013; 6: 756-762.
[21] Park H, Jung HY, Choi HJ, Kim DY, Yoo JY, Yun CO, Min JK, Kim YM and Kwon YG. Distinct roles of DKK1 and DKK2 in tumor angiogenesis. Angiogenesis 2014; 17: 221-234.
[22] Wang KP, Bai Y, Wang J and Zhang JZ. Morphine protects SH-SY5Y human neuroblastoma cells against Dickkopf1-induced apoptosis. Mol Med Rep 2015; 11: 1174-1180.
[23] Koppen A, Ait-Aissa R, Hopman S, Koster J, Haneveld F, Versteeg R and Valentijn LJ. Dickkopf-1 is down-regulated by MYCN and inhibits neuroblastoma cell proliferation. Cancer Lett 2007; 256: 218-228.
[24] Granchi D, Baglio SR, Amato I, Giunti A and Baldini N. Paracrine inhibition of osteoblast differentiation induced by neuroblastoma cells. Int J Cancer 2008; 123: 1526-1535.
[25] Granchi D, Corrias MV, Garaventa A, Baglio SR, Cangemi G, Carlini B, Paolucci P, Giunti A and Baldini N. Neuroblastoma and bone metastases: clinical significance and prognostic value of Dickkopf 1 plasma levels. Bone 2011; 48: 152-159.
[26] Lou Qian CW, Shi J Li Y, Wang D, Zhang PH, Li P. Clinical significance of CSF-DKK1 as a biomarker for diagnosis and therapeutic monitoring of neuroblastoma. Journal of Third Military Medical University 2013; 5.