RESEARCH COMMUNICATION

Relationships between Serum Biomarker Levels and Clinical Presentation of Human Osteosarcomas

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Abstract

Background: Currently, serum biomarkers play an important role as sensitive tools for monitoring the cancer development and progression. Each biomarker represents a specific pathogenesis and has different predictive capability. In order to identify their characteristics in human osteosarcoma, multiple potential biomarkers were analyzed simultaneously with clinical presentations. Materials and Methods: Blood samples were collected from 28 osteosarcoma patients and 30 healthy matched controls. Specific clinical presentations were recorded, including: tumor volume, estimated based on three-dimensional MRI volumetric measurement; metastasis status; and histological cell types. Serum biomarkers analyzed by ELISA-based assays were bone-specific alkaline phosphatase (BALP), vascular endothelial growth factor (VEGF), hyaluronic acid (HA) and chondroitin sulfate WF6 (WF6). Serum lactate dehydrogenase (LDH) was analyzed by a photometric-based system. Results: Serum BALP, LDH and WF6 levels of osteosarcoma patients were significantly higher than those of healthy controls, whereas HA and VEGF levels were not significantly different between the two groups. Serum BALP and LDH were positively correlated with tumor volume, (correlation coefficients 0.5 and 0.4, respectively). Serum BALP from metastasis and osteoblastic subtype group had a significantly higher level than that found in non-metastasis and non-osteoblastic subtypes group, respectively. Upon multivariate analysis, tumor volume was the only factor which correlated with BALP levels. Conclusion: Of the biomarkers analyzed in this study, serum BALP was the most reliable and sensitive for estimating tumor volume. A high level of serum WF6 reflects alteration of the extracellular matrix component of tumors. Both serum biomarkers can be expected to be further explored for use in specific clinical monitoring.

Keywords: Bone-specific alkaline phosphatase - osteosarcoma - tumor volume - VEGF - chondroitin sulfate - WF6

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Introduction

Osteosarcoma is the most common malignant bone tumor in adolescents and young adults. Although multidisciplinary approaches and limb-sparing surgeries have improved the survival rate and quality of life, a better understanding of the tumor growth and metastasis is still required for improving the future treatment strategies. Currently, serum biomarkers play an important role to be a sensitive tool for monitoring the pathogenesis of disease because they are the relation between released biomakers from tumors and dynamic body distribution and excretion (Sawyers, 2008). Serum biomarker level is not always related to an expression level in local tissue. Therefore, the validation studies between serum biomarkers and specific clinical presentation would be performed before applying each biomarker for clinical interpretation. Multiple biomarker study moreover can provide a comparative view for selecting the most potential biomarkers within the specific clinical situation.

The poor prognosis of osteosarcoma depends on patient- and treatment-related factors. The patient-related factors directly associate to the pathogenesis of disease, which affects to serum biomarker levels. Tumor volume, histological type, and metastasis status at initial presentation have been known as the patient-related factors predicting the survival rate of osteosarcoma (Bacci et al., 2006). Tumor volume determines the number of cancer cells and growth potential of the tumor in host. The tumor volume at initial presentation, and volume change after chemotherapy, are known to be significant determinants of the prognosis (Moon et al., 2005). The initial metastasis implies the systemic burden of cancer cells, and the ability of cancer cells to escape from the

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host's local and systemic defenses (Nguyen et al., 2009). Previous studies showed that the overall survival rate significantly decreases in patients with initial metastasis (Harting et al, 2006; Rasalkar et al, 2011). The histological subtype of osteosarcoma represents cellular differentiation based on genetic background, autocrine and paracrine effects in local tissues, and systematic body responses. Histological subtype was also proven to be a significant predictor of the chemotherapeutic response and survival prognosis (Hauben et al., 2002).

Herein, three important patient-related poor prognostic factors were studied in relation to the potential biomarkers. Bone-specific alkaline phosphatase (BALP) relates to osteoblastic activity. Lactate dehydrogenase (LDH) is an enzyme involving anaerobic cancer metabolism. Vascular endothelial growth factor (VEGF) involves the potential of neovascularization. The extracellular matrix (ECM) component was monitored by chondroitin sulfate epitope WF6 (WF6) and hyaluronic acid (HA) levels.

Materials and Methods

General characteristics of study population

Blood samples were collected from 28 osteosarcoma patients and 30 healthy matched controls - during December 2008 to March 2010 at Chiang Mai University Hospital. This study was approved by the Research Ethics Committee of Chiang Mai University, and all subjects gave their consent before recruitment. Inclusion criteria for osteosarcoma patients included: (a) clinical and histological diagnosis of primary osteosarcoma as determined by the multidisciplinary musculoskeletal oncology team; and (b) first diagnosis without prior treatment. Patient-related prognosis factors - including tumor volume, initial metastasis and histological subtypes - were recorded. All patients received standard treatment and follow-up. The healthy control group was comprised of 30 cases matched for age and gender. Exclusion criteria consisted of: (a) history of recent musculoskeletal injury within three years; (b) abnormal liver and kidney function; (c) preexisting disease; and (d) currently taking medication for any reason.

Tumor volume measurement

Software used to estimate tumor volume was based on the multidisciplinary clinicians' delineation of the tumor boundary in each MRI image slice, using the following criteria: (1) normal anatomic structure from inhomogeneous tumor tissue; (2) any abrupt changes in signal between normal and tumor tissue; and (3) significant enhancement of gadolinium (Shin et al., 2000). The MRI parameters of images were varied patient by patient: i.e. the size was either 256 × 256 or 512 × 512 pixels; the numbers of slices ranged from 24 to 46; the resolutions in x-axis and y-axis ranged from 0.27 to 1.25 mm; and the spaces between slices ranged from 4.4 to 12 mm. These measurements were determined automatically by the software from the Digital Imaging and Communications in Medicine Headers. To take care of the possibility of broken boundaries, the binary mathematical morphological operators were applied. The basic morphological operations involving an image S and a structuring element E are:

dilation: $S \oplus E \in S\}$, erosion: $S \Theta E = \cup \{S - e: e \in E\}$, where $\cap$ and $\cup$ denote the set intersection and union, respectively.

$A + x$ denotes the translation of a set A by a point x, i.e.

$A + x = \{a + x: a \in A\}$.

After all slices of each patient's MRI images were processed, the total number of voxels was estimated by

$$V_{voxel} = \sum_{i=1}^{N} \sum_{y=1}^{n_y} \sum_{x=1}^{n_x} B_i(x, y) \quad ,$$

where Bi(x,y) is the value of pixel (x,y) in the boundary image B corresponding to the MRI image slice i, N is the total number of slices, nx and ny are the number of pixels in x-axis and y-axis, respectively. The tumor volume was finally estimated by

$$V = V_{voxel} \times r_x \times r_y \times s_{slice}$$

where rx, ry, and s_{slice} are the resolutions in x-axis and y-axis, and the spaces between slices, respectively. Three-dimensional tumor creation from an MRI is shown in Figure 1.

Biomarker analysis

Serum was collected from both groups between 8:00 a.m. and 10:00 a.m. prior to any treatments. Serum was centrifuged at 6,500 g for 10 min; supernatants were then stored at -80 °C until assayed. Healthy controls were advised to avoid any vigorous activities, including sports and running, for 2 d before blood collecting.

Bone-specific alkaline phosphatase: Serum BALP was determined by an immunoenzymatric assay (Ostase BAP, immunodiagnostic System Ltd, USA) Briefly, human serum was added into the assigned well on 96-well plate, followed by adding biotinylated anti-BAP. After incubation, the wells were then washed and peroxidase conjugated anti-biotin antibody was added and incubation. The reaction was stop by adding Quench reagent. An absorbance was determined using a microplate reader at 492/690 nm. The concentration of BALP in serum samples was calculated by reference to a standard curve.

Lactate dehydrogenase: Serum LDH was determined on photometric system performed in the clinical chemistry laboratory of Maharaj Nakorn Chiang Mai Hospital, on a Beckman analyzer from Backton Dickinson Diagnostics. For data analysis, the upper limit of normal of LDH was defined as 480 U/L.

Vascular endothelial growth factor: Serum VEGF was determined by an ELISA (Biotrak™ human VEGF ELISA, GE Healthcare, UK). Briefly, sample diluent was added into the assigned well on anti-human VEGF precoated stripwell plate and followed by serum. After incubation, the wells were then washed and biotinylated anti-human VEGF was added and then incubated. The streptavidin-HRP reagent was added and the plate was incubated. After washing, the detection of conjugated

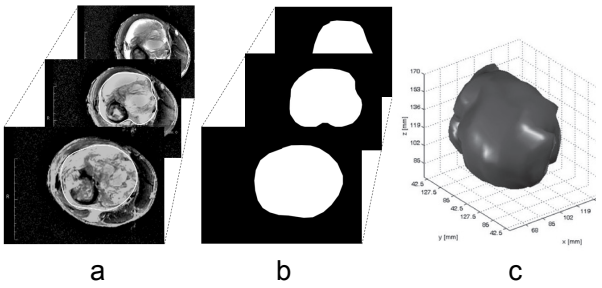

Figure 1. Method for Tumor Volume Estimation. Each pixel on an MRI is represented by 24 bits containing three values: red, green and blue (8 bits for each). (a) The boundaries were delineated in green (shown as light gray), and could be identified by detecting green pixels. A pixel would be considered as a part of a boundary if the result of its green value subtracted by its red value was greater than 100. A boundary image was generated with the same size as the original image. The pixels belonging to the boundary were set to 1, whereas the remaining pixels were set to 0. (b) Tumor areas in the x-y plane are automatically extracted. Each boundary image was first dilated to make sure that the boundary was completely closed; and then the enclosed pixels were set to 1. Next, erosion was applied. In this research, a disk structuring element with a radius of 5 pixels was applied to both dilation and erosion operations. (c) A three-dimensional tumor image was created following the method described

streptavidin was performed with TMB substrate. After stopping the enzymatic reaction with 1% sulfuric acid, absorbance was determined using a microplate reader at 450 nm. The concentration of VEGF in serum samples was calculated by reference to a standard curve.

Hyaluronic acid: HA was determined by ELISA-based assay using biotinylated HA-binding proteins (HABPs). Briefly, human serum samples were added to plastic tubes containing biotinylated HABPs. After incubation, the tubes were added to the microplate, precoated with umbilical cord HA and blocked with 1% BSA. The wells were then washed and peroxidase-conjugated antibiotin antibody was added. Detection of conjugated antibody was performed with ortho-phenylenediamine. The concentration of HA in samples was calculated from the standard curve (Pothacharoen et al., 2006).

Chondroitin sulfate WF6: Serum chondroitin sulfate WF6 was determined by a competitive immunoassay with a monoclonal antibody, and a quantitative ELISA modified from a previous study (Pothacharoen et al, 2007). Briefly, diluted human serum samples were added to plastic tubes containing an equal volume of WF6. They were added to the microtitre plate pre-coated with shark aggrecan (A1 fraction). Non-specific protein binding was blocked with BSA. The wells were then washed and peroxidase-conjugated anti-mouse IgM antibody was added. The bound conjugate was detected by adding ortho-phenylenediamine substrate. The reaction was stopped by 4M sulfuric acid, and absorbance was determined using a microplate reader at 492/690 nm. The concentration of WF6 epitope in supernatant samples was calculated by reference to a standard curve.

Statistical analysis

Serum levels of BALP, LDH, VEGF, WF6 and HA of osteosarcoma patients and healthy controls were compared using the Mann-Whitney U test. The correlation between tumor volume and biomarker levels used Pearson's correlation analysis. Subgroup analysis included metastasis at initial diagnosis; and histological subtypes were analyzed using Mann-Whitney U test. Multivariate analysis between biomarker levels and clinical presentations used a three-way ANOVA test. All statistical analyses were performed using SPSS software version 16. Statistical significance was defined as $p < 0.05$.

Results

Identification data

The 28 osteosarcoma patients (18:10 male/female) and 30 healthy controls (20:10) shared similar values of basic laboratory parameters including age, white blood cell count, body mass index, serum creatinine, AST and ALT levels, whereas the hemoglobin levels among osteosarcoma patients were lower. 78.6% of tumors located in the extremities - distal femur (81.8%), proximal tibia (9.1%) and the humerus (9.1%) - and 21.4% located at axial skeletal.

According to the histology, 89.2% were classified as conventional subtypes (71.4% osteoblastic, 10.7% chondroblastic and 7.1% fibroblastic), and 10.7% were classified as non-conventional osteosarcoma (7.1% giant

Table 1. Identification Data of Osteosarcoma Patients and Healthy Controls

Data	Osteosarcoma (28)	95% CI	Control (30)	95% CI	p-value
Age (year)§	15 yrs (7-53)	14-20	16 yrs (15-25)	16-18	0.051
Hemoglobin (g/dL)‡	12.5 (1.88)	11.8-13.2	14 (0.12)	13.7-14.2	< 0.05*
White blood cells (cell/uL)‡	9,551 (2,551)	8,562-10,541	9,100 (91)	8,914-9,286	0.58
Body mass index (kg/m2)‡	18.2 (2.8)	17-19.3	19.2 (0.44)	18.3-20.1	0.83
Serum creatinine (mg/dL)‡	1.07 (1.2)	0.6-1.5	0.83 (0.3)	0.77-0.9	0.81
Serum BUN(mg/dL)‡	11.28 (3.6)	9.9-12.6	10 (0.07)	9.8-10.1	0.33
AST (IU/L)‡	36.28 (45.3)	18.7-53.8	34.4 (8)	18-50.8	0.83
ALT (U/L)‡	28.82 (43.8)	11.8-45.8	30 (7.6)	14.4-45.7	0.45
BALP(U/L)¶	154 (30.3)	91.9-216.4	19 (1.58)	15.8-22.2	< 0.05*
LDH(U/L)¶	408.6 (58.4)	288.7-528.4	111.6 (19.5)	71.8-151.5	< 0.05*
WF6(ng/mL)¶	650.3 (126.8)	390.2-910.4	259.5 (28.7)	200.8-318.1	0.034*
HA(ng/mL)¶	90.9 (32)	25.3-156.5	43.3 (7.9)	27.1-59.5	0.20
VEGF (ng/mL)¶	174.8 (21.4)	131-218.6	146.5 (18)	109.7-183.2	0.16

§values are the median(range); ‡ values are the mean(SD); ¶ values are the median (SE); *statistically significant

cell-rich cell type, and 3.6% telangiectatic cell type). The details are further described in Table 1.

Serum biomarker levels between osteosarcoma patients and controls

Serum BALP, LDH and WF6 levels of osteosarcoma patients were significantly higher than those of healthy controls, whereas serum HA and VEGF levels were not significantly different between the two groups.

In ROC curve analysis, for discriminating between osteosarcoma patients and healthy match controls (Figure 2), the cutoff value of BALP was 34.3 U/L (sensitivity 0.78 and specificity 0.94), LDH was 151.5 U/L (sensitivity 0.82 and specificity 0.97 U/L), and WF6 was 394 ng/mL (sensitivity 0.54 and specificity 0.17).

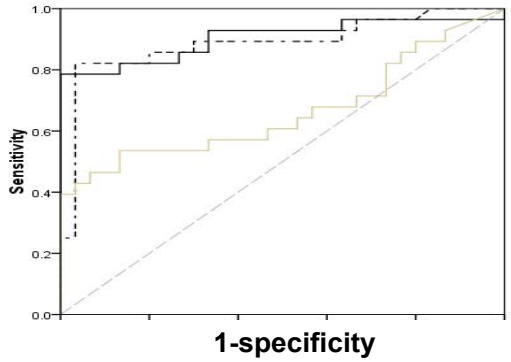

Figure 2. Receiver-operating Characteristic (ROC) Curves Constructed from Serum BALP, LDH and WF6, Distinguishing between Osteosarcoma Patients and Healthy Controls

Relationships between serum levels of biomarkers and clinical presentations

A simple correlation between serum BALP, LDH and WF6 levels and tumor volume showed that serum BALP and LDH were positively correlated with tumor volume, correlation coefficients of 0.5 and 0.4, respectively (Figure 3a,b).

Serum BALP level from metastasis status at initial diagnosis showed a significantly higher level than that of the non-metastasis group, whereas LDH and WF6 levels were not shown to have any significant difference (Figure 3d-f). Serum BALP from the osteoblastic (conventional) subtype showed a significantly higher level than that of the non-osteoblastic subtype, whereas LDH and WF6 levels were not shown to have any significant difference (Figure 3g-i).

Multivariate correlation analysis between serum BALP, LDH and clinical presentations were carried. Tumor volume was classified into two groups: large volume (> 200 mL3) and small volume (< 200 mL3). The tumor volume was the only factor which correlated to the BALP level ($p < 0.05$), and there was no correlation observed in the data between LDH and features of clinical presentation.

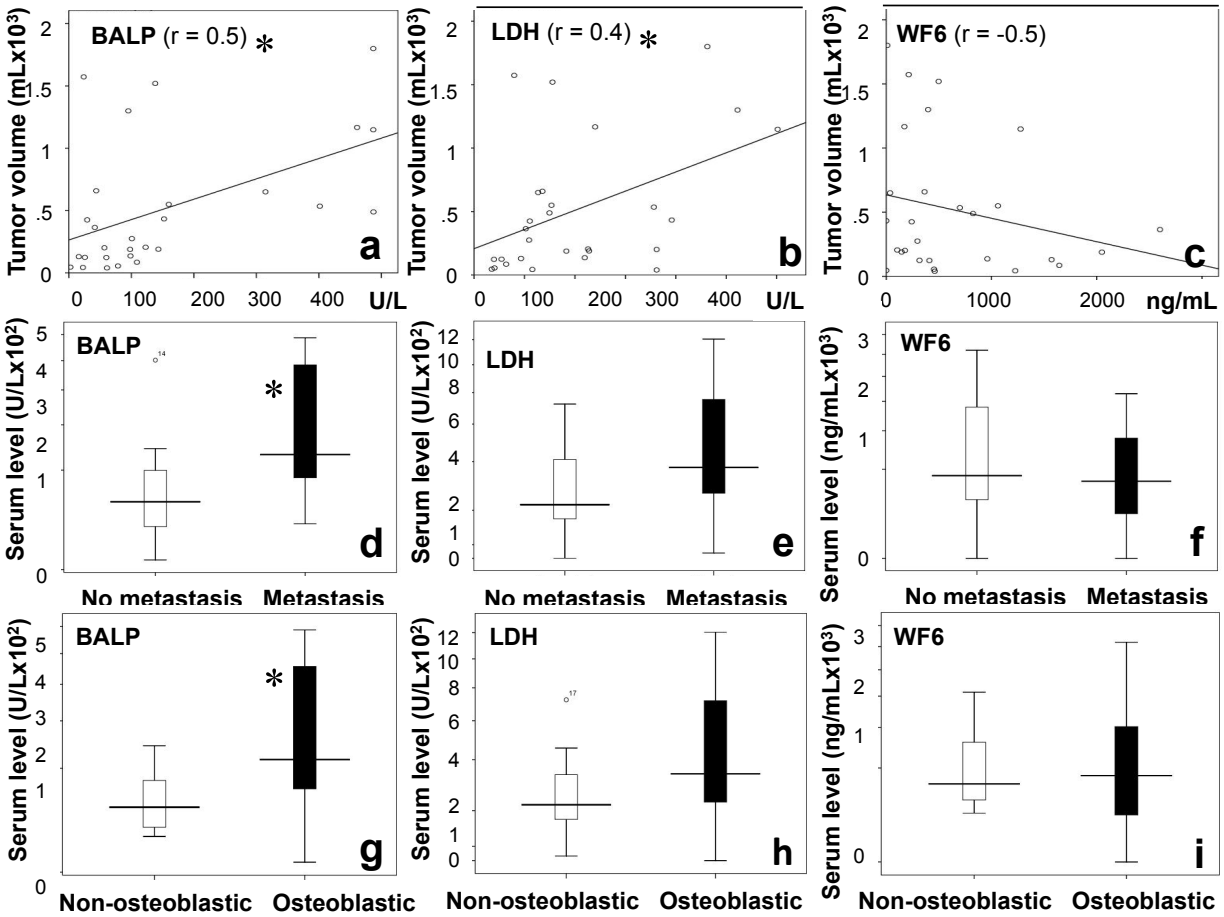

Figure 3. Relationships between Serum BALP, LDH and WF6 and Clinical Presentations of Osteosarcomas. (a-c) Simple correlation analysis between tumor volume and biomarker levels. Bar graphs show the median values of biomarker levels in each subgroup: (d-f) metastasis status at initial diagnosis; (g-i) and histological subtype (*$p < 0.05$)

Discussion

Serum BALP and LDH have been known to involve in the pathogenesis of tumor development on different aspect. Bone-specific alkaline phosphatase isozyme, a member of the zinc metalloproteinase family, is produced from and located on the cell membrane of osteoblasts. Its activity involves dephosphorylation of an extracellular inorganic pyrophosphate which regulates the matrix maturation and mineralization of bone (Leung et al, 1993). Transformed osteoblasts in osteosarcoma disrupt the tight control of proliferation, and progressively express the genes associated with cell differentiation, causing a constantly high level of the local BALP (Stein et al, 1990). In the other hand, LDH is an enzyme involved in cancer metabolism. The uncontrollable proliferation causes rapidly increasing tumor volume, and leads to insufficient nutrient and oxygen supply at the central area. A hypoxic microenvironment and hyperproliferation of cancer cells speeds up an anaerobic metabolism in order to obtain an adequate energy supply (Pruksakorn et al, 2010). According to the result, the correlation of tumor volume and serum LDH level in our study represented the higher metabolic demand of the tumor, as opposed to levels found in normal situations, which also could be found in other solid tumors and hematologic malignancies (Tas et al, 2001; Garcia et al, 1993). Serum BALP seemed to have a better specificity to clinical factors of osteosarcoma than those of serum LDH including a better degree of correlation between serum levels and tumor volume, a significant different of serum levels on metastasis status and histological type due to the simple correlation analysis. Moreover, serum BALP showed the greatest potential to estimate the tumor volume which could determine the number of transformed osteoblasts in the primary tumor and cancer colonies in the systemic burden. Therefore, a constantly high or progressively higher serum BALP level would be considered for aggressive investigation during the course of follow-up. The further quantitative validation of serum level would be performed in order to use serum BALP assisting in disease monitoring.

Angiogenesis is a consequence of rapid growth and hypoxic response of cancer cells. The high expression of VEGF in tissue correlates with the high density of microvascular in a biopsy sample, and relates to metastasis status (Kaya et al, 2002). Currently, VEGF become a potential therapeutic target and a poor prognostic marker for osteosarcoma progression (Rossi et al, 2010; Ek et al, 2006; Lin et al, 2010). Nevertheless, serum VEGF level still has an inconclusive role in disease monitoring. A higher serum VEGF levels (> 1,000 pg/mL) in osteosarcoma had a worse prognosis for survival than did lower serum VEGF levels (< 1,000 pg/mL) (Kaya et al, 2009); however, another study did not show any significant difference of serum VEGF levels between 17 osteosarcoma patients and healthy controls (Holzer et al, 2001). The results of this study, corresponding to the previous report, also did not show any significant difference of serum VEGF levels between the two groups. Although local VEGF expression has been studied to show a strong relationship with the neovascularization status, based on current evidences, the circulating VEGF was unable to discriminate the neovascularization between osteosarcoma patients and healthy controls.

According to our study, serum chondroitin sulfate levels showed a significant difference between the two groups. These differences reflect the significant changes in ECM metabolism which could be recognized by the circulating antibody. Chondroitin sulfate-proteoglycans (CS-PGs) are important extracellular matrix components along with cell surface glycoproteins. The interaction of CS-PGs and effecter molecules plays a role in cancer progression and metastasis (Liu et al, 2002; Timar et al, 2002)). Alterations in the glycosaminoglycan component regulate variety of cellular characteristics including proliferation and differentiation (Nikitovic et al, 2006). Recently, several reports have studied CS-PGs as anti-cancer targets (Fuster et al, 2005; Misra et al, 2003). The recognition of circulating CS in osteosarcomas would be helpful in further studies to predict ECM modification in osteosarcoma. Hyaluronic acid also has been studied for its local effect on osteosarcoma in vitro. An increase of endogenous HA production and exogenous high-molecular weight HA supplement of the osteosarcoma cell line correlates with the migration capability (Berdiaki et al, 2010; Tofuku et al, 2006). However, this study did not show a significant difference of circulating HA level between the two groups. Future studies might focus on local tissue rather than on the circulating level.

Among the biomarkers in this study, serum BALP proved to be the most reliable and sensitive for estimating tumor volume. Further benefits may be derived from quantitative studies of BALP level as a tool for better detection of the early progression of tumor metastasis, and of serum WF6 level as a biomarker for monitoring extracellular matrix metabolism during the course of treatment and over the follow-up period.

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