Prognostic factors for pulmonary metastasis in primary osteosarcoma

Eunah Shin

Department of Medicine

The Graduate School, Yonsei University

Prognostic factors for pulmonary metastasis in primary osteosarcoma

Directed by Professor Woo Ick Yang

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

Eunah Shin

December 2008

This certifies that the Doctoral Dissertation of Eunah Shin is approved.

Woo Ick Yang, M.D., PhD.

Jin-Suck Suh, M.D., PhD.

Kyeongmee Park, M.D., PhD.

Sun Young Rha, M.D., PhD.

Kyung-A Lee, M.D., PhD.

The Graduate School
Yonsei University

December 2008

ACKNOWLEDGEMENTS

I am most grateful to Professor Woo Ick Yang and Professor Sun Young Rha for their patience and guidance throughout my journey to the degree. I am also greatly indebted to Professor Jin-Suck Suh, Professor Kyeongmee Park and Professor Kyung-A Lee for their kind and meticulous instructions.

I express my deepest gratitude to my loving husband, Dr. Gijong Yi, and my most beloved sons Gene W. and Howon, who are indeed the greatest joy of my everyday life. I am also grateful to my parents-in-law who have shown me with their lives what patience and devotion mean.

To my dearest friends Dr. Cheryn Song, Dr. Kyungwon Kim and Dr. Jaseung Koo, I give thanks simply for just being there for me.

I thank my brother Jaehyeok for bearing with me all the time and lastly, I dedicate this thesis to my loving parents whom I respect the most in every aspect of life and who have been more than fully supportive throughout my life to this day.

December, 2008

Eunah Shin

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Prognostic factors for pulmonary metastasis in primary osteosarcoma

Eunah Shin

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Woo Ick Yang)

Osteosarcoma is the most common primary tumor of bone with a 5-year survival of approximately 70%, which drops to 37% once the tumor recurs or metastasizes. Thus, identifying prognostic factors for pulmonary metastasis can be one of the keys to improving the overall survival of osteosarcoma. In an endeavor to identify novel factors of prognostic significance, a panel of immunohistochemistry and fluorescent in situ hybridization (FISH) studies were done in tissue microarray of 92 cases of osteosarcoma, 26 of which were tumors with metachronous pulmonary metastasis. Additionally, to newly discover differentially expressed genetic molecules between metastatic and non-metastatic osteosarcoma tissues, cDNA microarray was done in 8 fresh frozen tissues of osteosarcoma, 4 of which were tumors with metachronous pulmonary metastasis. The immunohistochemical studies were comprised of metastasis-related molecules in osteosarcoma previously screened by cDNA microarray studies such as ezrin, vascular endothelial growth factor (VEGF), and nm23. Antibodies for EGFR, c-kit, nerve growth factor receptor (NGFR), nuclear factor kappa B (NFκB), tyrosine receptor kinase (Trk) A, B, and C, and HER-2/neu were stained additionally. The fluorescent in situ hybridization studies were comprised of HER-2/neu, epidermal growth

factor receptor (EGFR), and c-MET. When the immunohistochemical staining results were correlated with clinical variables, nm23 and NFκB were correlated with the histologic type of the tumor. Ezrin and VEGF were significantly correlated with the pulmonary metastasis of the tumor and nm23, EGFR, c-kit, NGFR, Trk-A, B, and C, and HER-2/neu showed no statistical significance. In the metastatic group of 24 cases, 21 cases (87.5%) showed immunoreactivity to ezrin and 16 cases (66.7%) showed positive reaction to VEGF. In survival analyses, VEGF negative cases had better disease-free survival rate when compared to the VEGF positive cases with statistical significance. Although statistically non-significant, there was a trend for better survival rate for ezrin-negative cases in comparison with the ezrin-positive cases. There were no amplified signals for HER-2/neu, EGFR, c-MET in fluorescent in situ hybridization, precluding the possibility of targeted drug therapy in osteosarcoma by means of verification of chromosomal amplification. Although the same cannot be said for EGFR and c-MET, the totally negative result in the FISH for HER-2/neu is in concordance with the immunohistochemical stain result, which also showed totally negative staining for HER-2/neu. In an attempt to newly discover genetic molecules that are differentially expressed between the metastatic and non-metastatic osteosarcoma tissues, cDNA microarray study revealed 5 totally new molecules (OSTM1, LYSMD3, C3orf63, NMI, PSMB8) with more than two-fold change in the metastatic osteosarcoma. The newly found molecules are not overlapping with those previously found in literature review. Further studies for validation of these molecules are necessary.

Key words : osteosarcoma, lung metastasis, ezrin, VEGF, nm23, prognostic factor, cDNA microarray

Prognostic factors for pulmonary metastasis in primary osteosarcoma

Eunah Shin

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Woo Ick Yang)

I. INTRODUCTION

Osteosarcoma, the most common primary tumor of bone, is the second highest cause of cancer-related death in the pediatric age group.[1] The principal treatment modality consists of neoadjuvant chemotherapy followed by limb-salvage operation. However, despite the introduction of adjuvant chemotherapy with resultant tumor necrosis of considerable extent, the 5-year survival is approximately 70% and has not changed much over the last 20 years.[1,2] Moreover, this figure drops to 40%-50% if the chemotherapy induced tumor necrosis falls short of 90% and even more dramatically to 37% once the tumor recurs or metastasizes. Such inferior chemotherapy induced necrosis has been correlated with higher rates of pulmonary metastasis, which is an overriding determinant of survival.[3] Approximately 20% of the patients show lung metastasis at initial diagnosis and 30% to 40% eventually develop metastasis in spite of the conventionally established treatment.[4] There are some distinctive features of metastasis in osteosarcoma, such as 1) long latent period between initial diagnosis of the primary tumor and the development of metastasis, 2) preference of lung as metastatic site, 3) relative success associated with surgical removal of the metastasis.[5] Consequently, there have been many endeavors to identify predictive factors for pulmonary metastasis and novel markers of therapeutic and prognostic importance for this highly debilitating and fatal entity, but unfortunately the results have been controversial, if not futile, and have

resulted in the plateau of survival rate for more than 20 years.[6] Recently, owing to the fast developing biotechnology and tumor biology, identification of newly emerging molecular markers for prognostic significance and development of new therapeutic targets have been possible. Moreover, drugs specifically targeted to inhibit certain genetic alteration involved in oncogenesis and tumor progression have been developed and have led to improved outcomes for the patients especially when combined with multiagent chemotherapy. Namely such targeted drug therapies include trastuzumab, which is specific for HER-2/neu amplification in breast cancers, gefitinib, which is a specific inhibitor of epidermal growth factor receptor (EGFR), and imatinib mesylate, inhibitor of KIT activity.[7-9] Since their successful clinical utility that was more than expected, studies exploring the possibility of their application to various other tumors have been continued and osteosarcoma has been no exception. However, studies involving osteosarcoma to date have been limited by a small number of index cases or cell line studies only. For instance, Morris et al. has reported that HER-2/neu expression is associated with significantly less tumor necrosis after preoperative chemotherapy, and thus antibodies that target this receptor should be considered for the treatment.[10] The shortcoming is, though, that their study has involved immunohistochemical staining only and the true gene amplification has not been investigated and correlated.

The primary aim of this study is to identify factors which can be predictive of higher metastatic potential of a given osteosarcoma, so that the patients with lower chemosensitivity and higher metastatic potential can be subject to alternative chemotherapeutic regimen earlier enough to improve overall survival. To this end, various markers with established prognostic and therapeutic significance in other solid tumors and molecules previously screened by cDNA microarray studies to be associated with metastatic potential in osteosarcoma are applied immunohistochemically to a large series of osteosarcoma in tissue microarray and the results are analyzed with appropriate statistical methods. In addition, the differential protein expressions of erbB family protein receptor

tyrosine kinases, HER-2/neu and EGFR, in osteosarcoma with and without pulmonary metastases are analyzed immunohistochemically and the alterations at genetic level are subsequently explored and correlated by fluorescent in situ hybridization (FISH), in an attempt to investigate the possibility of targeted drug therapy in osteosarcoma in addition to conventional chemotherapy.

The secondary aim of this study is to define, by cDNA microarray, novel genetic determinants that are differentially expressed in the tumors that develop pulmonary metastasis and the tumors that do not.

II. MATERIALS AND METHODS

1. Construction of tissue microarray

A total of 105 archival paraffin-embedded osteosarcoma tissues from 1993 through 2004 were retrieved from the Department of Pathology, Yonsei University College of Medicine. They were classified into metastatic group who developed pulmonary metastases during follow-up period and non-metastatic group. Upon review of the slides, representative areas of viable tumor were marked and from these areas, two 2mm-sized cores for each case were punched from the paraffin blocks with a precision instrument and arrayed on a recipient paraffin block (Figure 1). Unstained slides of 4-um sections of these tissue microarray blocks were prepared for immunohistochemical studies and FISH.

2. Immunohistochemical stain

The unstained slides were deparaffinized in xylene, rehydrated in graded alcohols, and processed using the labeled streptavidin-biotin-peroxidase method. They were then submitted to heat-induced antigen retrieval for 15 minutes in 0.1M citrate buffer and subsequently incubated in 10% normal blocking serum for 30 minutes. They were incubated overnight at 4℃ in appropriately diluted primary antibodies. Mouse monoclonal antibody to ezrin (1:300; NeoMarkers, Fremont, CA, USA), goat polyclonal antibody to vascular endothelial growth

factor (VEGF) (1:150; R&D Systems, Minneapolis, MN, USA), rabbit polyclonal antibody to nm23 (1:700; NeoMarkers), ready to use antibody for EGFR using EGFR PharmDx™, rabbit polyclonal antibody to HER-2/neu (1:250; DAKO, Glostrup, Denmark), mouse monoclonal AE1/AE3 antibody for CK (1:100; DAKO), rabbit polyclonal antibodies for tyrosine receptor kinase-A (Trk-A), B, and C (1:200; Santa Cruz, Santa Cruz, CA, USA), rabbit polyclonal antibody to nuclear factor kappa B (NFκB) (1:200; NeoMarkers), rabbit polyclonal antibody to c-kit (1:30; DAKO), and mouse monoclonal antibody for low affinity nerve growth factor receptor (NGFR) (1:50; DAKO) were used for immunohistochemistry (Table 1). After washing with Tris buffer, sections were incubated with biotin-labelled secondary antibodies and then with streptavidin-horseradish peroxidase using the DAKO LSAB kit (DAKO) at room temperature for 30 minutes for each step. Nova red (Vector Laboratory, Burlingame, CA, USA) was used as the chromogen and hematoxylin as the nuclear counterstain. This procedure was performed for all antibodies under study except for EGFR. Ready to use antibody was employed for immunohistochemical stain of EGFR using EGFR PharmDx™ , and the staining procedure was done as the manufacturer's protocol.

Table 1. Antibodies used for immunohistochemical stain

1° Antibody	Origin	Dilution factor	Company
ezrin	mouse monoclonal	1:300	NeoMarkers
VEGF	goat polyclonal	1:150	R&D Systems
nm23	rabbit polyclonal	1:700	NeoMarkers
HER-2/neu	rabbit polyclonal	1:250	DAKO
CK	mouse monoclonal	1:100	DAKO
Trk-A, B, and C	rabbit polyclonal	1:200	Santa Cruz
NFκB	rabbit polyclonal	1:200	NeoMarkers
c-kit	rabbit polyclonal	1:30	DAKO
NGFR	mouse monoclonal	1:50	DAKO
EGFR			EGFR PharmDx™

3. Fluorescent in situ hybridization

FISH assays for HER-2/neu (Vysis Inc., Downers Grove, IL, USA), EGFR (ZytoVision, Bremerhaven, Germany), and c-MET (Vysis Inc.) were performed using commercial probes following manufacturer's protocol. Histology FISH accessory kit (DAKO) was used for detection. Briefly, the prepared tissue slides were deparaffinized to remove paraffin and rehydrated at room temperature. The deparaffinized and rehydrated slides were immersed and incubated for 10 minutes in preheated (98℃) Pre-Treatment Solution. After cooling in room temperature for 15 minutes, washing steps were repeated twice. Ready-to-use pepsin drops were applied on the slides, incubated for 10 minutes at room temperature, and soaked in the diluted Wash Buffer for 3 minutes at room temperature. Afterwards, the tissue sections were dehydrated through a graded series of ethanol: 2 minutes in 70% ethanol, 2 minutes in 85% ethanol,

and 2 minutes in 96% ethanol, and then air dried completely. An appropriate amount of the probe was applied to the slides, covered with glass coverslip overlapped by Cover slip Sealant to form a seal around the coverslip, and placed in Dako Hybridizer. The denaturation was set to 72 °C for 10 min and hybridization to 37 °C overnight. Washing and dehydrating steps were repeated and the slides were counterstained with blue fluorescence counterstain Fluorescence Mounting Medium.

The number of signals of chromosome 17 for HER-2/neu and chromosome 7 for EGFR and c-MET, signals for each probe, and the number of tumor nuclei scored were recorded for each core. At least 30 tumor nuclei were counted per tissue core, and the signal enumeration was performed under x1000 magnification using epifluorescence microscope with single-interference filter sets for green (FITC), red (Texas red), and blue (DAPI) as well as triple (blue, red, green) band pass filters. Tumors were interpreted as amplified when the ratio of target gene signals to centromere (CEP) 17 or CEP 7 signals was $\geq$2.0. The average ratio of different cores from the same tumor was used as the final score for the determination of gene amplification status of that particular tumor.

4. cDNA microarray

A. RNA extraction and amplification

Total RNA was extracted from fresh frozen tissues, which were immediatedly frozen at -80℃ at the time of incisional biopsy for initial diagnosis, by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The Yonsei reference RNA (Cancer Metastasis Research Center, Seoul, Korea) was prepared by pooling equivalent amounts of total RNA from 11 human cancer cell lines. The quantity and quality of RNA were confirmed by a ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA) and gel electrophoresis. First-strand template cDNA was generated by reverse transcription with an oligo$(dT)_{15}$ primer coupled to a T7 RNA polymerase recognition sequence (Applied Biosystems, Framingham, MA,

USA). Residual single-stranded RNA was removed by RNase H digestion and the cDNA template was used for transcription with biotinylated CTP and UTP nucleosides to produce a cRNA template. After purification and quantification, the biotinylated cRNA was fragmented by hydrolysis, producing 35 to 200 nucleotide segments.

B. Hybridization

Following the institutional protocol, samples were hybridized at 45℃ to a human cDNA chip (CMRC-GT, Seoul, Korea) containing ~17000 cDNA clone of 300bp~3kb with a reference design. The test samples were labeled with Cy5 and individually co-hybridized with the Cy3-labeled Yonsei reference RNA (CMRC, Seoul, Korea).

C. Preprocessing and data analysis

For further analysis, raw Cy5/Cy3 data were log2-transformed. Systemic errors were corrected by normalization using intensity dependent, within-print, tip normalization based on the Lowess function. After normalization, genes with more than one missing value in all experiments were filtered, no missing proportion (NMP) 80%. The values of repeated genes were adjusted by S-Plus 2000 software (Insightful, Seattle, WA, USA). We determined the significant genes which could divide the tissues into metastatic and non-metastatic groups using two-class significance analysis of microarrays (SAM)[11] with selected genes. Hierarchical clustering analysis was performed with Cluster (Eisen Lab, http://rana.lbl.gov/EisenSoftware.htm) and the resulting dendrogram was visualized using TreeView software (Eisen Lab). Clustering was done by complete linkage algorithm with uncentered correlation. The distance of each cluster represents correlation between two clusters. Annotation of the selected genes was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (http:// apps1. niaid. nih. gov/ david) and the Stanford Online Universal Resource for Clones and Expressed Sequence Tags (SOURCE) (http:// source. stanford. edu/ cgi-bin/ source/ source Search).

5. Collection of clinical data

Clinical information were collected from the medical chart review and the clinicopathologic factors assessed for prognostic significance included age, gender, site of involvement, histologic subtype of the tumor, percentile of chemotherapy induced necrosis, recurrence, follow-up duration and survival.

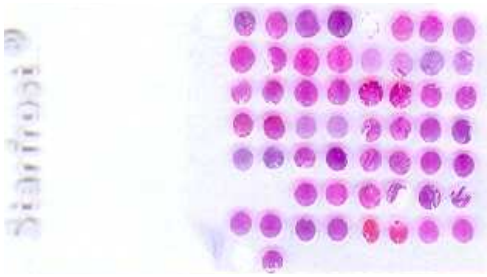
6. Statistical analysis

Sets of statistical analysis based on the aims of the study to investigate factors associated with increased risk of developing pulmonary metastasis were done. Univariate analysis and multivariate analysis were performed to assess prognostic significance and value of individual risk factors. In all statistical analyses, a two-tailed p-value < 0.05 was considered statistically significant. All analyses were performed using SPSS for Windows statistical software (Version 15.0)

III. RESULTS

1. Tissue microarray construction results

Of the selected 105 cases of osteosarcoma 32 cases were osteosarcomas with metachronous lung metastasis and 73 cases were devoid of lung metastasis neither at the time of initial diagnosis nor during the follow-up period. All slides were reviewed and representative tumor areas were marked appropriately. Cases with insufficient amount of tissue in paraffin blocks and those with missing slides or blocks were dropped. The total number of evaluated cases was 92 in toto, 66 of which comprised the non-metastatic group and 26 of which belonged to the metastatic group. After tissue microarray construction, there were tissue cores from each group either insufficient for evaluation or lost upon serial sectioning. Therefore, the total number of evaluated cases differed for each antibody or probe (Figure 1).


A.

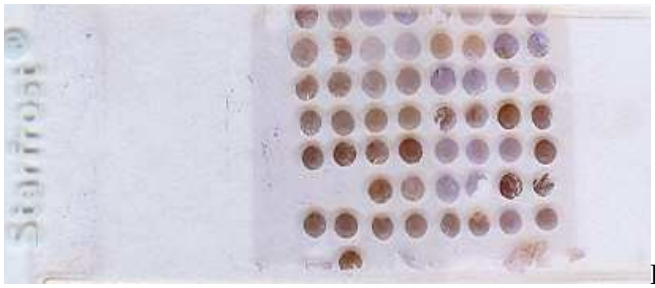

B.

Figure 1. Representative image of tissue microarray slides. A) H&E stain, B) Immunohistochemical stain

2. Patient demographics

Of the total number of 92 cases, 48 were male and 44 were female with a mean age of 21.6 years (range 3 to 65 years). Thirty-five cases were respondent to chemotherapy when 'responsiveness' is defined as chemotherapy induced tumor necrosis more than 90% and 37 were not responsive, while chemoresponsiveness in 20 cases were not assessed due to lack of post-chemotherapeutic specimen. Chemotherapeutic regimen consisted of either combination of adriamycin and intra-arterial cisplatin or combination of ifosfamide, adriamycin and intra-arterial cisplatin. Regarding the location of the tumor, 71 cases were tumors arising in the lower extremity, 7 cases were in the upper extremity, 8 cases were tumors arising in sites other than upper and lower extremities. Six cases had no records on site of involvement. As for the histologic subtype, 51 cases were osteoblastic type, 8 were chondroblastic, 4 were fibroblastic, 12 were mixed (of which osteoblastic mixed with chondroblastic type was the most common), 7 cases were parosteal/ periosteal, and 10 cases were other rare subtypes including intramedullary well differentiated, small cell, pleomorphic, and telangiectatic

subtypes, and extraskeletal osteosarcoma (Table 2, Figure 2).

Table 2. Patient demographics

Clinical variables	Subclassification	Results
Sex		F:M=44:48
Age		21.6 (range3–65)
Site	upper extremity	7 (7.6%)
	lower extremity	71 (77.2%)
	other	8 (8.7%)
	not assessed	6 (6.5%)
Histologic type	parosteal/periosteal	7 (7.6%)
	osteoblastic	51 (55.4%)
	chondroblastic	8 (8.7%)
	fibroblastic	4 (4.3%)
	mixed	12 (13.0%)
	other	10 (10.7%)
Response to chemotherapy	>90%	35 (38.0%)
	<90%	37 (40.0%)
	Not assessed	20 (22.0%)
Pulmonary metastasis	no metastasis	66 (71.7%)
	metastasis	26 (28.3%)

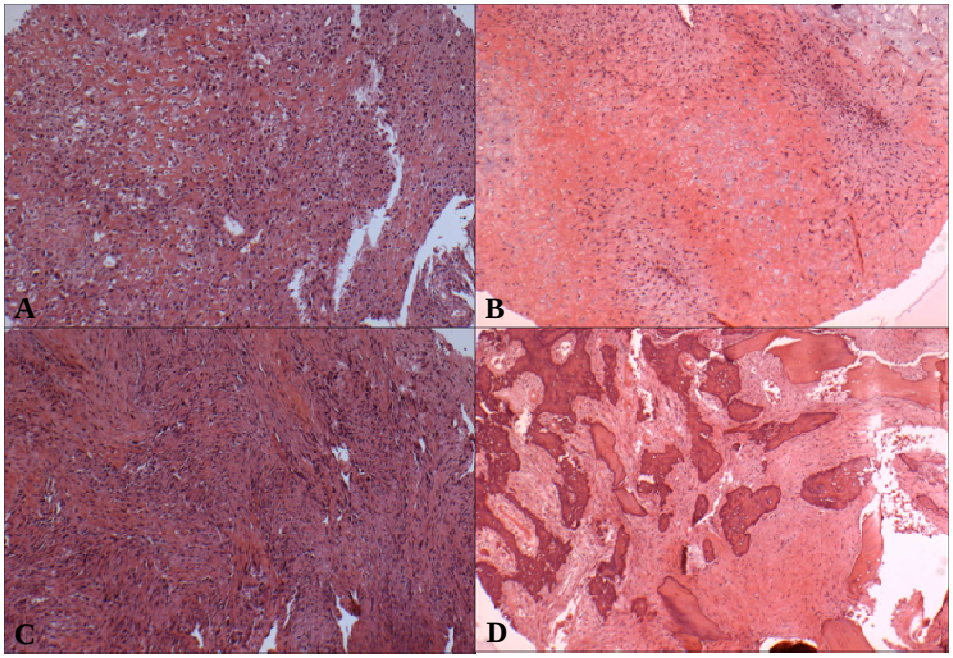


Figure 2. Histologic subtypes of osteosarcoma (H&E, x40). A) osteoblastic type, B) chondroblastic type, C) fibroblastic type, D) intramedullary well- differentiated type.

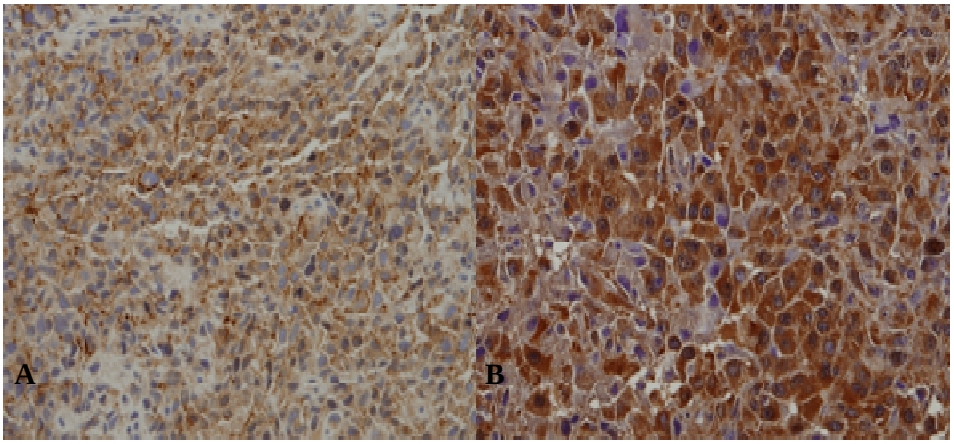


Figure 3. Immunohistochemical stains for A) ezrin and B) nm23, showing membranous staining for ezrin and cytoplasmic staining for nm23 (x100).

3. Various protein expression in osteosarcoma by immunohistochemical stain

As aforementioned, the total number of evalulated cases ranged from 78 to 92 due to lost cores during tissue microarray construction and serial sectioning. For ezrin, nm23, c-kit, Trk-A, Trk-B, and NFκB, more than 50% of the cases were immunoreactive, whereas VEGF and EGFR showed relatively low expression rates of 42% and 25%, respectively. NGFR was predominantly non-immunoreactive in index cases (Figure 2). None of the cases showed immunoreactivity for Trk-C, CK, and HER-2/neu (Table 3).

Table 3. Immunohistochemical stain results in primary osteosarcoma

Protein	Positive ratio
ezrin	47/83 (57%)
nm23	57/83 (69%)
c-kit	59/83 (71%)
Trk-A	64/78 (82%)
Trk-B	66/78 (85%)
NFκB	49/79 (62%)
VEGF	33/79 (42%)
EGFR	23/92 (25%)
NGFR	3/92 (3%)
Trk-C	0 /78 (0%)
CK	0 /78 (0%)
HER-2/neu	0 /78 (0%)

4. Fluorescent in situ hybridization results

All cases evaluated for amplification of HER-2/neu, EGFR, and c-MET showed no amplified signal in osteosarcoma tissues.

5. Correlation of protein expression and clinical variables

When each immunohistochemical staining result was evaluated for correlation with clinical variables other than lung metastasis, nm23 and NFκB were positively correlated with osteoblastic subtype of the tumor (p-value 0.023 and 0.015, respectively) and no other antibody was correlated with any of the clinical variables (Table 4).

Table 4. p-values for the correlation of immunohistochemical stain results and clinical variables

	Sex	Age	Site	Histologic Type	Response to chemotherapy
ezrin	0.059	0.0586	0.395	0.093	0.166
VEGF	1.00	0.1560	0.687	0.272	0.145
nm23	0.871	0.1233	0.403	*0.023*	0.151
EGFR	0.603	0.8755	0.961	0.447	0.778
c-kit	0.122	0.4983	0.831	0.551	0.99
NGFR	0.621	0.3382	1.000	0.132	0.608
Trk-A	0.206	0.3133	0.450	0.518	0.489
Trk-B	0.792	0.2775	0.202	0.711	0.712
NFκB	0.531	0.4178	0.511	*0.015*	0.214

Ezrin expression was evaluated in 83 cases, of which 24 were with lung metastasis. Among 59 non-metastatic tumors, 26 (44.1%) were positive for ezrin and 21 out of 24 (87.5%) were positive for ezrin in lung metastasis group (p-value 0.0003).

Nm23 was evaluated in 83 cases, and of the 60 non-metastatic tumors, 38 (63.3%) were positive for nm23 and 19 out of 23 metastatic tumors (82.6%) were positive for nm23.

For EGFR, 13 out of 66 (19.7%) were immunoreactive in the non-metastatic group and 10 out of 26 (38.5%) were immunoreactive in the metastatic group. For NGFR evaluated in a total of 92 patients, only one case from the non-metastatic group and two cases from the metastatic group showed positive immunostaining.

Immunohistochemical staining for Trk-A and Trk-B showed similar results for both metastatic and non-metastatic groups, with 83.3% (45 out of 54) and 85.2% (46 out of 54) respectively in non-metastatic group and 79.2% (19 out of 24) and 83.3% (20 out of 24) respectively in metastatic group.

For VEGF and NFκB, cores of 79 cases were available. In the metastasis group, 16 out of the 24 (66.7%) were immunoreactive to VEGF and in the non-metastasis group, only 17 out of the 55 (30.9%) were immunoreactive (p-value 0.003). As for NFκB, 15 out of the 24 (62.5%) were immunoreactive in the metastasis group and 34 out of the 55 (61.8%) were immunoreactive in the non-metastasis group, which were statistically not significant.

Immunohistochemical stain results for Trk-C, CK, and HER-2/neu were totally negative in all cases evaluated. These immunohistochemical staining results, except for those of ezrin and VEGF, were all statistically insignificant (Table 5).

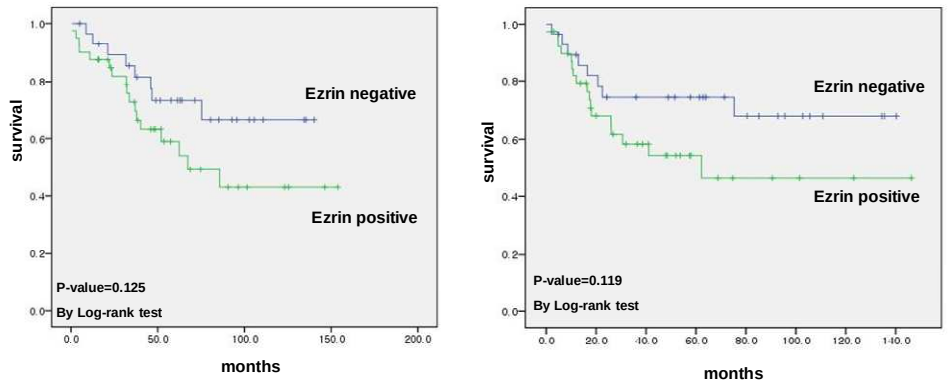
Table 5. Correlation of protein expression and pulmonary metastasis

Protein expression		Pulmonary metastasis		p-value
		Present (%)	Absent (%)	
Ezrin	Positive	21 (87.5)	26 (44.1)	*0.0003*
	Negative	3 (12.5)	33 (55.9)	
VEGF	Positive	16 (66.7)	17 (30.9)	*0.003*
	Negative	8 (33.3)	38 (69.1)	
nm23	Positive	19 (82.6)	38 (63.3)	0.0902
	Negative	4 (17.4)	22 (36.7)	
EGFR	Positive	10 (38.5)	13 (19.7)	0.0613
	Negative	16 (61.5)	53 (80.3)	
C-kit	Positive	19 (79.2)	40 (67.8)	0.3754
	Negative	5 (20.8)	19 (32.2)	
NGFR	Positive	2 (7.7)	1 (1.5)	0.1915
	Negative	24 (92.3)	65 (98.5)	
Trk-A	Positive	19 (79.2)	45 (83.3)	0.7516
	Negative	5 (20.8)	9 (16.7)	
Trk-B	Positive	20 (83.3)	46 (85.2)	0.999
	Negative	4 (16.7)	8 (14.8)	
NFκB	Positive	15 (62.5)	34 (61.8)	0.954
	Negative	9 (37.5)	21 (38.2)	

6. Survival analyses according to ezrin and VEGF expressions.

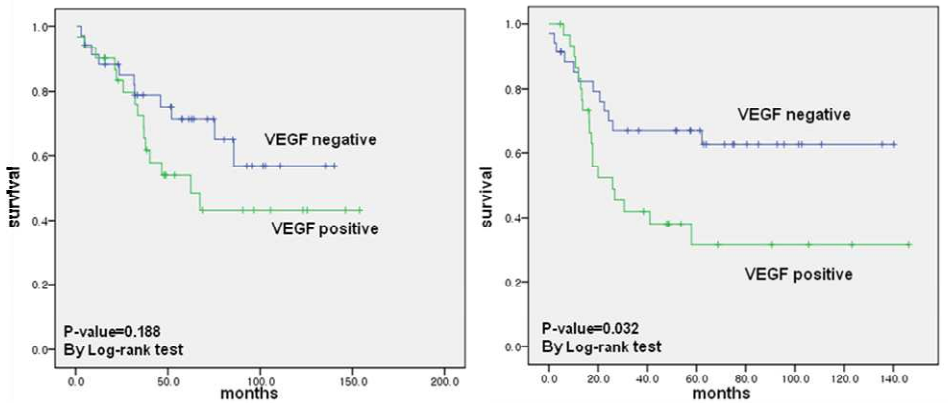
The overall survival and disease-free survival in relation to the ezrin overexpression were evaluated by log-rank test. The median follow-up duration was 90 months. The median in overall survival was 77.25 months (range 0.6 – 153.9) and the median in disease-free survival was 74.25 months (range 2.2 –

146.3). Although there was no statistical significance, there was a trend for higher survival curve for those that were ezrin-negative (Figure 3). Meanwhile, VEGF negative group showed higher disease-free survival rate in contrast to the VEGF positive group with a statistical significance (Figure 4).



A. **B.**

Figure 4. A) Overall survival and B) disease-free survival in ezrin-positive and ezrin-negative osteosarcoma



A. **B.**

Figure 5. A) Overall survival B) disease-free survival in VEGF-positive and VEGF-negative osteosarcoma

7. Coexpressions of ezrin, VEGF, nm23, and EGFR

In addition, we evaluated the coexpressions of 4 molecules (ezrin, VEGF, nm23, and EGFR) to see whether the coexpressions were correlated to the outcome of osteosarcoma. There was no correlation between the coexpressions and chemoresponsiveness or disease-free survival. However, lung metastasis was positively correlated with 5 combinations of coexpressions and only that of ezrin and nm23 showed statistical significance for overall survival (Table 6).

Table 6. Correlation of pulmonary metastasis, chemoresponsiveness, overall survival, and disease-free survival with coexpressions of ezrin, VEGF, nm23, and EGFR

Ezrin and VEGF	Pulmonary Metastasis		Response to Chemotherapy		Overall Survival		Disease-free Survival	
	Present	Absent	≥90%	<90%	≥24m	<24m	≥24m	<24m
+/+	14	14	14	10	18	8	13	13
+/−, −/+	9	15	7	9	15	4	11	8
−/−	2	26	8	16	18	5	16	7
p-value	*0.0018*		0.218		0.687		0.379	

Ezrin and nm23	Pulmonary Metastasis		Response to Chemotherapy		Overall Survival		Disease-free Survival	
	Present	Absent	≥90%	<90%	≥24m	<24m	≥24m	<24m
+/+	7	24	18	15	24	12	20	16
+/−, −/+	6	16	8	9	28	1	12	7
−/−	2	17	3	12	11	4	10	5
p-value	*0.049*		0.081		*0.048*		0.721	

Ezrin and EGFR	Pulmonary Metastasis		Response to Chemotherapy		Overall Survival		Disease-free Survival	
	Present	Absent	≥90%	<90%	≥24m	<24m	≥24m	<24m
+/+	10	9	8	6	15	2	10	7
+/−, −/+	11	21	12	12	15	10	14	11
−/−	3	29	9	18	55	5	18	9
p-value	*0.003*		0.278		0.073		0.719	

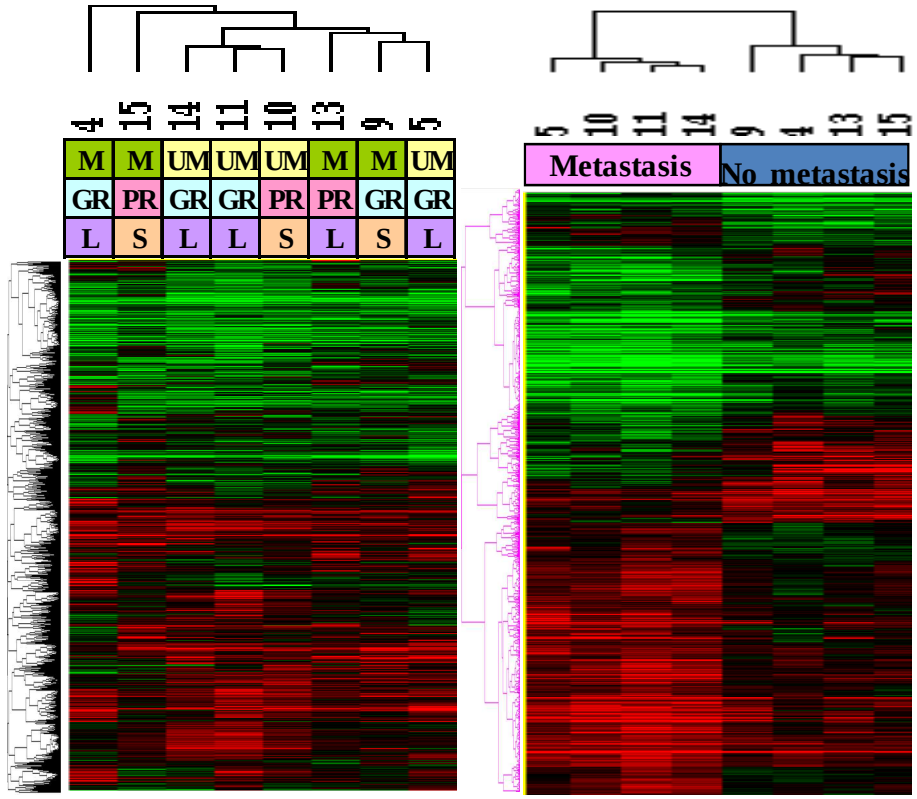
VEGF and nm23	Pulmonary Metastasis		Response to Chemotherapy		Overall Survival		Disease-free Survival	
	Present	Absent	≥90%	<90%	≥24m	<24m	≥24m	<24m
+/+	13	15	15	11	19	8	13	14
+/−, −/+	9	25	11	14	23	5	19	9
−/−	2	15	3	10	8	4	7	5
p-value	*0.0399*		0.121		0.473		0.334	

VEGF and EGFR	Pulmonary Metastasis		Response to Chemotherapy		Overall Survival		Disease-free Survival	
	Present	Absent	≥90%	<90%	≥24m	<24m	≥24m	<24m
+/+	7	3	5	3	9	1	5	5
+/−, −/+	12	24	15	15	22	8	17	13
−/−	6	28	9	17	20	8	18	10
p-value	*0.0068*		0.297		0.488		0.696	

nm23 and EGFR	Pulmonary Metastasis		Response to Chemotherapy		Overall Survival		Disease-free Survival	
	Present	Absent	≥90%	<90%	≥24m	<24m	≥24m	<24m
+/+	7	11	8	8	13	2	10	5
+/−, −/+	15	29	18	15	29	11	22	18
−/−	3	20	5	13	13	4	10	7
p-value	0.122		0.176		0.651		0.736	

8. cDNA microarray results

Eight fresh frozen tissues acquired at the time of the incisional biopsy for initial diagnosis were available for cDNA microarray analysis, 4 of which belonged to the metastatic group and the remaining 4 in the non-metastatic group. Seven hundred and eighty-three genes were differentially expressed with a p-value of less than 0.05 (Figure 6). Five of these genes identified were upregulated greater than two-fold in the metastasis group (Table 7) and six were downregulated with fold change less than 0.6 (Table 8).



A. **B.**

Figure 6. Hierarchial clustering of cDNA microarray. A) Unsupervised clustering of 15354 genes. B) Supervised clustering of metastatic and non-metastatic osteosarcoma tissues with 783 genes.

Table 7. List of upregulated genes in metastasis group

Biologic function	Gene title	Symbol	Fold change
Degradation of G-protein	osteopetrosis associated transmembrane protein 1	OSTM1	2.01
Cell wall catabolic process	Not given	LYSMD3	2.15
Unknown	Not given	C3orf63	2.25
Interaction with N-Myc and C-Myc	N-Myc interactor and STAT interactor	NMI	3.00
Multicatalytic proteinase complex	proteasome subunit 8, prosome, macropain	PSMB8	3.67

Table 8. List of downregulated genes in metastasis group

Biologic function	Gene title	Symbol	Fold change
GTPase activator	Regulator of G-protein signaling	RGS3	0.26
Extracellular matrix structural constituent in cartilagenous tissue	Aggrecan/versican proteoglycan family	ACAN	0.27
Endocytosis, brain development and formation of bone	Alpha2-HS glycoprotein	AHSG	0.46
Changes in cell shape, motility or function	Wiskott-Aldrich syndrome protein family	WASF2	0.51
Chemoattractant activity	Macrophage inflammatory protein-1	CCL3	0.54
		RCAN2	0.56

IV. DISCUSSION

Putative prognostic markers for osteosarcoma, especially those that are expected to predict pulmonary metastasis, searched to date are many.[12,13] However, several studies employing different methodologies report different results even for the same markers. The most consistent marker of all that have been tried is the cytoskeleton linker protein ezrin, which is involved in cell adhesion, regulating the growth and metastatic capacity of cancer cells.[14] It has been identified as a metastasis-associated gene in cancer by cDNA microarray and since then it has been nominated as a crucial factor for determining metastatic potential of osteosarcoma.[15-18] Recently, its mRNA expression has been reported to be 5 times higher in a tumor sample with lung metastasis compared to the samples without metastasis.[19] However, its implication in metastatic potential of osteosarcoma needed to be validated by a larger number of index cases. Although this study has only evaluated the differential expression between the osteosarcomas with lung metastasis and those without by means of immunohistochemistry, significant difference has been demonstrated in the expression rate of ezrin between the two groups. According to Park et al., ezrin immunoreactivity is present in 43.7% of high-grade osteosarcoma specimens and all low-grade osteoarcomas are negative for ezrin.[20] This result has implicated that ezrin immunoreactivity may also be increased in the osteosarcomas with higher potential for pulmonary metastasis and our result confirmed the implication. Nonetheless, the expectation that there must be other prognostic markers that can predict pulmonary metastasis in osteosarcoma by significantly different level of expression between the two groups has been somewhat disappointed. However, in addition to ezrin, VEGF has been shown to be differentially expressed in osteosarcomas with lung metastasis and those without. VEGF, although well-known to be involved in angiogenesis and hence endowed with a relatively important role in the early settlement of metastatic clones, has not been fully investigated in human osteosarcoma. It has been reported to be associated with tumor growth in osteosarcoma cell line and the latest reported result on VEGF in relation to the metastatic potential of osteosarcoma was by

Park et al., which stated that VEGF expression in osteosarcoma is not different between the metastatic and non-metastatic groups.[21,22] However, their research comprised of only two cases of metastatic osteosarcoma and the number is rather small to be sufficient for validated conclusion. My result shows significantly increased expression of VEGF in the metastatic group, in compliance with the expectation that it must be increased in the metastatic tumors when its angiogenetic role and the importance of angiogenesis in metastasis are taken into account. Also it may have to do with the fact that the osteosarcoma is highly vascularized tumor and the expression of VEGF is the booster to the metastatic potential of the already vascular-rich tumors. According to the previous research, it may additionally be presumed that the angiogenetic role of VEGF in osteosarcoma is mainly for the growth or expansion of the primary tumor mass rather than for the establishment of metastatic clones, but this of course has to be corroborated in human tissue study in the near future. A contrasting result to the hypothesis is the increased expression of nm23 in the metastatic group though there was no statistical significance. nm23 is a putative metastasis-suppressor gene originally identified in highly metastatic murine melanoma cells.[23] Its reduced expression is associated with higher rate of lymph node metastasis in breast carcinomas and its expression is reduced at the metastatic site of gastric and colorectal carcinomas.[24] Although recent data have failed to show any inverse relationship between nm23 expression and metastatic potential in breast cancers, a positive correlation between nm23 and metastatic capacity has been shown in rat osteosarcomas.[25] Although the paradoxical result of more frequent nm23 expression in metastatic tumors showed no statistical significance, Oda et al. had reported similar result with statistical significance.[26] They evaluated 25 cases of metastatic osteosarcoma with both primary tumor and metastatic tumor tissues available, and the result was that the nm23 expression was significantly higher in the metastatic tumor tissues than in the primary tumor tissues. Honoki et al has shown by Northern blotting that the level of nm23 was increased in rat-transplantable osteosarcoma cell lines that had higher metastatic potential.[27] Thus by review of the literature, my result regarding nm23 is also suggesting

that nm23 expression in osteosarcoma is, in contrast with the epithelial tumors, positively correlated with metastatic potential.

Some salient features of metastatic osteosarcoma, lung as the preferred site and the success associated with surgical removal of the metastatic tumor for instance, are somewhat reminiscent of epithelial malignancies[28] and hence tried were a few novel markers associated with epithelial and nerve growth factor receptors, namely cytokeratin, low-affinity NGFR (p75), Trk-A, Trk-B, and Trk-C. These markers have never been evaluated in osteosarcoma neither in cell line nor human tissues. The Trk proto-oncogene encodes a tyrosine kinase protein of 140kDa.[29] It is a member of a receptor tyrosine kinase family including related genes Trk-B and Trk-C.[30] Tyrosine kinase proteins are signaling transmembrane receptors for neurotrophins of nerve growth factor family, which are essential for the differentiation and development of central and peripheral nervous systems.[31] Studies have demonstrated the presence of Trk proteins and nerve growth factors (NGF) in the smooth muscles of vessels in animal models.[31] Low-affinity NGFR (p75) is a member of the tumor necrosis factor receptor family. It may modulate binding of NGF to Trk-A.[32] Since osteosarcoma is a highly vascularized tumor and rich vasculature is a prerequisite for distant metastasis, the possibility of differential expression of Trk family and p75 in osteosarcoma groups with and without lung metastasis was explored and unfortunately, there was no significant difference between the two groups. While Trk-A and B are immunoreactive in a relatively high percentage of osteosarcomas and the expression rate is irrelevant of the presence of lung metastasis, Trk-C is totally negative in all cases and p75 is near-totally negative. This may shed some light on the relationship between Trk-C and p75 at molecular level and the different actions of Trk-A, B, and C.

Amplification study for HER-2/neu, EGFR, and c-MET by FISH revealed no amplification for all three. HER-2/neu proto-oncogene is located on human chromosome 17 and encodes a 185kDa transmembrane glycoprotein with tyrosine kinase activity.[33] Overexpression of HER-2/neu has been shown in a variety of human tumors, especially breast carcinomas in which its expression is

correlated with poor prognosis but renders target therapy by trastuzumab.[34] Many reports have suggested HER-2/neu overexpression as possible predictive factor for osteosarcoma therapy, but their results are controversial.[35-39] Some studies have demonstrated significant overexpression of HER-2/neu in early pulmonary metastases of osteosarcoma,[40] while some have correlated its overexpression with better survival of osteosarcoma patients.[41] Others did not observe any HER-2/neu expression in osteosarcoma at all.[42-43] The overexpression of EGFR is observed in non-small cell lung cancers and other solid tumors including breast, head and neck, colon, kidney, and ovarian cancers.[44] The EGFR gene mutation is correlated with favorable outcome and increased sensitivity to EGFR tyrosine kinase inhibitors in non-small cell lung cancers.[45-46] Although there are quite a few studies on erbB receptors in osteosarcoma, most of them are focused on HER-2/neu and little has been revealed on the EGFR status in osteosarcoma. The investigation of its potential prognostic value can be rewarding if it can only render osteosarcoma patients as candidates for anti-EGFR treatment strategies.[47] In addition, MET proto-oncogene is the receptor for cytokine hepatocyte growth factor/scatter factor (HGF-SF), a disulfide-linked heterodimer produced by mesenchymal cells, such as macrophages, endothelial cells, and fibroblasts.[48] The functions of HGF-SF are mediated by the c-MET tyrosine kinase receptor, which activate various intracellular signaling pathways.[49] Overexpression of HGF-SF or c-MET has been identified in a number of epithelial tumors and various sarcoma, including osteosarcoma.[50] c-MET expression is shown to be increased in metastatic lesions of osteosarcoma compared to primary lesions,[51] whereas another study has demonstrated negative correlation between c-MET expression and the development of bone metastases.[52] There are controversies regarding the prognostic role of the three markers in osteosarcoma. That HER-2/neu amplification results by FISH are known to be well-correlated with the protein expression demonstrated by immunohistochemistry taken into account, my results of HER-2/neu amplification study by FISH and immunohistochemistry for protein expression are concordant and therefore, it can be safely said that

osteosarcoma is not associated with HER-2/neu amplification at all. On the other hand, EGFR protein overexpression was shown by immunohistochemistry though statistically insignificant between the metastatic and non-metastatic groups, while no amplification was shown by FISH. This may implicate EGFR mutation of osteosarcomas,[53] not amplification as in non-small cell lung carcinomas, and further studies at molecular level are required to investigate possible applicability of the EGFR-targeted therapy. Even so, the total absence of amplification in all cases is not only contradictory to all the studies reported heretofore, but also it cannot be easily explained.

In search of more reliable and novel markers, cDNA microarray was employed to assess differential genetic expression profiles between the metastatic and non-metastatic osteosarcoma tissues. Five differentially upregulated novel genes with fold change more than 2.0 were identified. Gene expression profile studies are mostly in vitro studies employing osteosarcoma cell lines with high- and low-metastatic potential,[54] which is possibly the reason for the emergence of totally new genes in our study, and they have yielded many differentially upregulated novel genes associated with metastasis in osteosarcoma, among which are ezrin, c-MET, nm23, VEGF, galectin 3, and Ki67.[55] Therefore, our study has a meaning in two aspects. One is that we have confirmed the association of the above mentioned novel genes screened by microarray in osteosarcoma cell lines heretofore with metastatic potential and other clinical variables in a large number of human tissues, and the other is that we have discovered totally novel genes differentially expressed between fresh human tissues of osteosarcoma with and without pulmonary metastasis. In addition, when the microarray analyses were done to search differentially expressed genes between osteosarcoma with chemoresponsiveness (>90% preoperative chemotherapy induced necrosis) and osteosarcoma without chemoresponsiveness (<90% necrosis) (data not shown), there were some overlapping genes differentially expressed both in metastatic tumors and chemoresponsive tumors. These genes had no known function and they left us with obligation to explore and uncover in the near future their hidden functions

and associations with metastatic potential and chemoresponsiveness. Among the differentially expressed genes between the chemoresponsive and non-responsive osteosarcoma tissues was NFκB, the receptor activator of which had been reported to be expressed in osteosarcoma cell lines in vitro by Mori et al.[56] Though it was not among the genes differentially expressed between the metastatic and non-metastatic osteosarcoma tissues, immunohistochemical stain of NFκB was added to the panel of immunohistochemistry. Though it was not significantly associated with pulmonary metastasis in osteosarcoma, it was associated with the histologic subtype of the tumor. Further research into the molecule may reveal some insight into the understanding of the biology of osteosarcoma.

V. CONCLUSION

In an endeavor to identify novel factors of prognostic and therapeutic significance, a panel of immunohistochemistry and fluorescent in situ hybridization studies were done in tissue microarray of 92 cases of osteosarcoma, and cDNA microarray was done in 8 fresh frozen tissues.

When the immunohistochemical staining results were correlated with clinical variables, nm23 and NFκB were correlated with the histologic type of the tumor and other factors were not significantly correlated. Among the immunohistochemically stained antibodies, ezrin and VEGF were significantly correlated with the pulmonary metastasis of the tumor and the remaining showed no statistical significance. Significantly higher disease-free survival rate was shown in VEGF-negative cases in comparison with the VEGF-positive cases. Although statistically not significant, there was a trend for better survival rate for ezrin-negative cases in comparison with the ezrin-positive cases. There were no amplified signals for HER-2/neu, EGFR, and c-MET in fluorescent in situ hybridization, precluding the possibility of current HER-2/neu and EGFR targeted drug therapies and yet developing c-MET targeted therapy in osteosarcoma by means of verification of chromosomal amplification.

In an attempt to newly discover differentially expressed genetic molecules in the metastatic and non-metastatic osteosarcoma tissues, cDNA microarray study revealed 5 totally new molecules (OSTM1, LYSMD3, C3orf63, NMI, PSMB8) with more than two-fold change in the metastatic osteosarcoma. The newly found molecules are not overlapping with those previously found in literature review. Further studies for validation of these molecules will be necessary.

REFERENCES

1. Wang LL. Biology of osteogenic sarcoma. Cancer J 2005;1:294–305.

2. Hellm´en E, Moller M, Blankenstein MA, Andersson L, Westermark B. Expression of different phenotypes in cell lines from canine mammary spindle-cell tumours and osteosarcomas indicating a pluripotent mammary stem cell origin. Breast Cancer Res Treat 2000;6:197–210.

3. Witlox MA, van Beusechem VW, Grill J, Haisma HJ, Schaap G, Bras J. Epidermal growth factor receptor targeting enhances adenoviral vector based suicide gene therapy of osteosarcoma. J Gene Med 2002;4:510–6.

4. Hughes DPM, Thomas DG, Giordano TJ, McDonagh KT, Baker LH. Essential erbB family phosphorylation in osteosarcoma as a target for CI-1033 inhibition. Pediatr Blood Cancer 2006;46:614–23.

5. Kim MS, Song WS, Cho WH, Lee SY, Jeon DG. Ezrin expression predicts survival in stage IIB osteosarcomas. Clin Orthop Relat Res 2007;459:229–36.

6. Hasegawa S, Hirose T, Kudo E, Hizawa K, Usui M, Ishii S. lmmunophenotypic heterogeneity in osteosarcomas. Hum Pathol 1991;22:583–90.

7. Ferrari S, Zanella L, Alberghini M, Palmerini E, Staals E, Bacchini P. Prognostic significance of immunohistochemical expression of ezrin in non-metastatic high-grade osteosarcoma. Pediatr Blood Cancer 2008;50:752–6.

8. Kansara M, Thomas DM. Molecular pathogenesis of osteosarcoma. DNA Cell Biol 2007;26:1–18.

9. Somers GR, Ho M, Zielenska M, Squire JA, Thorner PS. HER2 amplification and overexpression is not present in pediatric osteosarcoma: a tissue microarray study. Pediatr Dev Pathol 2005;8:525‒32.

10. Morris CD, Gorlick R, Huvos AG, Heller G, Meyers PA, Healey JH.

Human epidermal growth factor receptor 2 as a prognostic indicator in osteogenic sarcoma. Clin Orthop Relat Res 2001;382:59–65.

11. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A. 2001; 98: 5116–21.

12. Rouzier R, Perou CM, Symmans WF, Ibrahim N, Cristofanilli M, Anderson K, et al. Breast cancer molecular subtypes respond differently to preoperative chemotherapy. Clin Cancer Res 2005;11:5678–85.

13. Khanna C, Khan J, Nguyen P, Prehn J, Caylor J, Yeung C, et al. Metastasis-associated differences in gene expression in a murine model of osteosarcoma. Cancer Res 2001;61:3750–9.

14. Yang HS, Hinds PW. Phosphorylation of ezrin by cyclin-dependent kinase 5 induces the release of Rho GDP dissociation inhibitor to inhibit Rac1 activity in senescent cells. Cancer Res 2006;66:2708–15.

15. Hunter KW. Ezrin, a key component in tumor metastasis. Trends Mol Med 2004;10:201–4.

16. Curto M, Andrea I. McClatchey AI. Ezrin… a metastatic detERMinant? Cancer Cell 2004;5:113–4.

17. Salas S, Bartoli C, Deville JL, Gaudart J, Fina F, Calisti A, et al. Ezrin and alpha-smooth muscle actin are immunohistochemical prognostic markers in conventional osteosarcomas. Virchows Arch 2007;451:999–1007.

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;10:182–6.

19. Ogino W, Takeshima Y, Mori T, Yanai T, Hayakawa A, Akisue T, et al. High level of ezrin mRNA expression in an osteosarcoma biopsy sample with

lung metastasis. J Pediatr Hematol Oncol 2007;29:435–9.

20. Park HR, Jung WW, Bacchini P, Bertoni F, Kim YH, Park YK. Ezrin in osteosarcoma: comparison between conventional high-grade and central low-grade osteosarcoma. Pathol Res Pract 2006;202:509–15.

21. Tsai JY, Aviv H, Benevenia J, Chang VT, Patterson F, Aisner S, et al. HER-2/neu and p53 in osteosarcoma:an immunohistochemical and fluorescence in situ hybridization analysis. Cancer Invest 2004;22:16–24.

22. Lipinski M, Braham K, Philip I, Wiels J, Philip T, Goridis C, et al. Neuroectoderm-associated antigens on Ewing's sarcoma cell lines. Cancer Res 1987;47:183–7.

23. Tee YT, Chen GD, Lin LY, Ko JL, Wang PH. NM23-H1: a metastasis-associated gene. Taiwan J Obstet Gynecol 2006;45:107–13.

24. Aryee D, Strobel T, Kos K, Salzer-Kuntschik M, Zoubek A, Veron M, et al. High nrn23-HI/NDPK-A expression in Ewing tumors: paradoxical immunohistochemical reactivity and lack of prognostic significance. Int J Cancer 1995;64:104–11.

25. Liao WM, Chiu KY, Li FB, Qiu JS, Han SY, Chow SP. Expression on nm23 protein in human osteosarcoma in relationship with early metastasis. Orthopedics 2000;2;1175–8.

26. Oda Y, Naka T, Takeshita M, Iwamoto Y, Tsuneyoshi M. Comparison of histological changes and changes in nm23 and c-MET expression between primary and metastatic sites in osteosarcoma: a clinicopathologic and immunohistochemical study. Hum Pathol 2000;31:709–16.

27. Honoki K, Tsutsumi M, Miyauchi Y, Mii Y, Tsujiuchi T, Morishita T, et al. Increased expression of nucleoside diphosphate kinase/n/w23 and c-Ha-ras mRNA is associated with spontaneous lung metastasis in rat-transplantable osteosarcomas. Cancer Res 1993;53:5038–42.

28. Okada K, Hasegawa T, Yokoyama R , Beppu Y, Itoi E. Osteosarcoma with cytokeratin expression: a clinicopathological study of six cases with an emphasis on differential diagnosis from metastatic cancer. J Clin Pathol 2003;56:742–6.

29. Montano X. p53 associates with trk tyrosine kinase. Oncogene 1997;15: 245–56.

30. Donovan MJ, Hempstead BL, Horvath C, Chao MV, Schofield D. Immunohistochemical localization of trk receptor protein in pediatric small round blue cell tumors. Am J Pathol 1993;143:1560–7.

31. Nogueira E, Navarro S, Pellin A, Llombart-Bosch A. Activation of TRK genes in Ewing's sarcoma Trk A receptor expression linked to neural differentiation. Diagn Mol Pathol 1997;6:10–6.

32. Fanburg-Smith JC, Miettinen M. Low-affinity nerve growth factor receptor (p75) in dermatofibrosarcoma protuberans and other nonneural tumors: a study of 1,150 tumors and fetal and adult normal tissues. Hum Pathol 2001;32:976–83.

33. Willmore-Payne C, Holden JA, Zhou H, Gupta D, Hirschowitz S, Wittwer CT, et al. Evaluation of HER-2/neu gene status in osteosarcoma by fluorescence in situ hybridization and multiplex and monoplex polymerase chain reactions. Arch Pathol Lab Med 2006;130:691–8.

34. Kumar VR, Nalini G, Nandita K, Sharma SC. Prognostic and predictive value of c-erbB2 overexpression in osteogenic sarcoma. J Cancer Res Ther 2006;2:20–3.

35. Anninga JK, van de Vijver MJ, Cleton-Jansen AM, Kristel PMP, Taminiau AHM, Nooij M, et al. Overexpression of the HER-2 oncogene does not play a role in high-grade osteosarcomas. Eur J Cancer 2004;40:963−70.

36. Gorlick R, Huvos AG, Heller G, Aledo A, Beardsley GP, Healey JH, et al.

Expression of HER2/erbB-2 correlates with survival in osteosarcoma. J Clin Oncol 1999;17:2781–8.

37. Zhou H, Randall RL, Brothman AR, Maxwell T, Coffin CM, Goldsby RE. HER-2/neu expression in osteosarcoma increases risk of lung metastasis and can be associated with gene amplification. J Pediatr Hematol Oncol 2003;25:27–32.

38. Maitra A, Wanzer D, Weinberg AG, Ashfaq R. Amplification of the HER-2/neu oncogene is uncommon in pediatric osteosarcomas. Cancer 2001;92:677–83.

39. Scotlandi K , Manara MC, Hattinger CM, Benini S, Perdichizzi S, Pasello M, et al. Prognostic and therapeutic relevance of HER2 expression in osteosarcoma and Ewing's sarcoma. Eur J Cancer 2005;41:1349–61.

40. Ferrari S, Bertoni F, Zanella L, Setola E, Bacchini P, Alberghini M, et al. Evaluation of p-glycoprotein, HER-2/ErbB-2, p53, and bcl-2 in primary tumor and metachronous lung metastases in patients with high-grade osteosarcoma. Cancer 2004;100:1936–42.

41. Akatsuka T, Wada T, Kokai Y, Kawaguchi S, Isu K, Yamashiro K, et al. ErbB2 expression is correlated with increased survival of patients with osteosarcoma. Cancer 2002;94:1397–404.

42. Thomas DG, Giordano TJ, Sanders D, Biermann JS, Baker L. Absence of HER2/neu gene expression in osteosarcoma and skeletal Ewing's sarcoma. Clin Cancer Res 2002;8:788–93.

43. Fellenberg J, Krauthoff A, Pollandt K, Delling G, Parsch D. Evaluation of the predictive value of Her-2/neu gene expression on osteosarcoma therapy in laser-microdissected paraffin-embedded tissue. Lab Invest 2004;84:113–21.

44. Hughes DPM, Thomas DG, Giordano TJ, Baker LH, McDonagh KT. Cell surface expression of epidermal growth factor receptor and Her-2 with nuclear

expression of Her-4 in primary osteosarcoma. Cancer Res 2004;64:2047–53.

45. Kersting C, Agelopoulos K, Schmidt H, Korsching E, August C, Gosheger G, et al. Biological importance of a polymorphic CA sequence within intron 1 of the epidermal growth factor receptor gene (EGFR) in high grade central osteosarcomas. Genes Chromosomes Cancer 2008;47:657‑64.

46. Kersting C, Gebert C, Agelopoulos K, Schmidt H, van Diest PJ, Juergens H, et al. Epidermal growth factor receptor expression in high-grade osteosarcomas is associated with a good clinical outcome. Clin Cancer Res 2007;13:2998–3005.

47. Freeman BB, Daw NC, Geyer JR, Furman WL, Stewart CF. Evaluation of gefitinib for treatment of refractory solid tumors and central nervous system malignancies in pediatric patients. Cancer Invest 2006;24:310–7.

48. Scotlandi K, Baldini N, Oliviero M, Di Renzo MF, Martano M, Serra M, et al. Expression of Met/hepatocyte growth factor receptor gene and malignant behavior of musculoskeletal tumors. Am J Pathol 1996;149:1209–19.

49. MacEwen EG, Kutzke J, Carew J, Pastor J, Schmidt JA, Tsan R, et al. C-Met tyrosine kinase receptor expression and function in human and canine osteosarcoma cells. Clin Exp Metastasis 2003;20:421–30.

50. Ferracini R, Angelini I, Cagliero E, Linari A, Martano M, Wunder P, et al. MET oncogene in canine aberrant expression of osteosarcoma. J Orthop Res 2000;18:253–6.

51. Cantiani L, Manara MC, Zucchini C, De Sanctis P, Zuntini M, Valvassori L, et al. Caveolin-1 reduces osteosarcoma metastases by inhibiting c-Src activity and Met signaling. Cancer Res 2007;67:7675‑85.

52. Coltella N, Manara MC, Cerisano V, Trusolino L, Di Renzo MF, Scotlandi K, et al. Role of the MET/HGF receptor in proliferation and invasive behavior of osteosarcoma. FASEB J 2003;17:1162–4.

53. Wen YH, Koeppen H, Garcia R, Chiriboga L, Tarlow BD, Peters BA, et al. Epidermal growth factor receptor in osteosarcoma:expression and mutational analysis. Hum Pathol 2007;38:1184–91.

54. Lisle JW, Choi JY, Horton JA, Allen MJ, Damron TA. Metastatic osteosarcoma gene expression differs in vitro and in vivo. Clin Orthop Relat Res 2008;466:2071–80.

55. Leonard P, Sharp T, Henderson S, Hewitt D, Pringle J, Sandison A, et al. Gene expression array profile of human osteosarcoma. Br J Cancer 2003;89:2284–8.

56. Mori K, Le Goff B , Berreur M, Riet A, Moreau A, Blanchard F, et al. Human osteosarcoma cells express functional receptor activator of nuclear factor-kappa B. J Pathol 2007;211:555–62.

ABSTRACT (IN KOREAN)

원발성 골육종의 폐전이에 대한 예측인자

<지도교수 양 우 익>

연세대학교 대학원 의학과

신 은 아

골육종은 뼈에 생기는 원발성 종양 중 가장 흔한 악성 종양으로서 5년 생존율이 약 70% 정도 되며 종양이 재발하거나 특히 폐전이가 되면 이러한 생존율은 약 37% 정도로 떨어진다. 따라서 폐전이를 예측할 수 있는 인자를 찾는 것이 골육종의 생존율을 높이는 데 있어 중요한 열쇠가 될 수 있겠다. 골육종의 예후와 치료에 있어서 중요한 의미를 가질 수 있는 새로운 인자를 찾기 위하여 원발성 종양의 진단 후 폐전이가 생긴 골육종 26 증례를 포함한 92개의 골육종 조직으로 tissue microarray를 만들어 면역염색과 fluorescent in situ hybridization (FISH) 을 시행하였다. 이 중 폐전이가 생긴 4개의 증례를 포함하여 신선 냉동 조직이 있는 총 8개의 증례에 대하여 폐전이가 있는 조직과 폐전이가 없는 조직에서 유의한 차이를 가지고 발현되는 새로운 유전인자를 찾기 위하여 cDNA microarray를 시행하였다. 면역조직화학 염색은 ezrin, VEGF, nm23 등 cDNA microarray 연구 결과 골육종에서 폐전이와 연관성이 있을 것으로 보고된 인자들에 대하여 시행하였고 fluorescent in situ hybridization은 HER-2/neu, EGFR, c-MET에 대하여 시행하였다. 면역조직화학 염색 결과와

대상군의 임상적인 특징을 비교분석한 결과 nm23과 NFκB가 종양의 조직학적 분류(osteoblastic type)와 유의하게 상관성이 있었으며 nm23, EGFR, c-kit, NGFR, Trk-A,-B,-C, HER-2/neu등 다른 인자들은 대상군의 임상적인 특성과 유의한 상관성이 없었다. 또한, 폐전이에 대한 비교분석에서는 ezrin과 VEGF가 종양의 폐전이에 대하여 통계학적으로 유의한 상관성을 보였고 다른 인자들은 폐전이와 유의한 상관성이 없었다. 이들 두 인자에 대한 생존율 분석을 시행하였을 때 각각에 대하여 음성인 군이 양성인 군에 비해 높은 생존율을 보였다. FISH 연구 결과 종양은 92증례 모두 HER-2/neu, EGFR, c-MET에 대한 증폭이 없는 것으로 나타나 골육종에서 HER-2/neu나 EGFR의 증폭에 따른 표적치료의 가능성을 기대하기는 어려울 것으로 생각된다. HER-2/neu에 대한 FISH 결과는 면역조직화학 염색 결과와 상관성이 매우 높다는 사실을 감안할 때 본 연구에서 HER-2/neu에 대한 면역조직화학 염색 결과가 92증례 모두에서 음성으로 나온 것은 FISH 결과와 일치한다고 볼 수 있다. 폐전이가 있는 군과 없는 군에서 유의한 차이를 가지고 발현되는 새로운 인자를 찾기 위하여 시행한 cDNA microarray 연구 결과 5개의 새로운 인자가 (OSTM1, LYSMD3, C3orf63, NMI, PSMB8) 폐전이가 있는 조직에서 2배 이상 높게 발현되었다. 이들 5개의 인자는 기존의 골육종 연구 결과 폐전이와 관련이 있을 것으로 밝혀진 인자들과는 다른 새로운 인자들로서 앞으로 이들 인자의 발현을 확인할 수 있는 연구가 필요할 것으로 생각된다.

핵심되는 말: 골육종, 폐전이, ezrin, VEGF, nm23, cDNA microarray