Int J Clin Exp Pathol 2016;9(11):12154-12170
www.ijcep.com /ISSN:1936-2625/IJCEP0037328

Original Article

Clinical and histopathological features of patients with metastatic solid tumor of unknown origin in bone marrow: an eight-year follow-up study

Qiong Feng[1*], Gao-Rong Deng[2*], Rui Gong[3*], Liang Hao[4], Zhi-Qiang Gong[5], Jian Sun[3], Xiao-Liang Lou[3], Xue-Feng Yu[3], Nong-Rong Wang[3], Lv Zhou[6], Jin-Ping Hu[6], Xiao-Feng Huang[6], Xiao-Qing Qi[6], Yan-Juan Deng[6], Huan Deng[3,5,6]

Departments of [1]Pathology, [4]Osteology, The Second Affiliated Hospital of Nanchang University, Nanchang, China; [3]Molecular Medicine and Genetics Center, Departments of [2]Osteology, [6]Pathology, The Fourth Affiliated Hospital of Nanchang University, Nanchang, China; [5]Renmin Institute of Forensic Medicine in Jiangxi, Nanchang, China. [*]Equal contributors.

Received August 4, 2016; Accepted August 23, 2016; Epub November 1, 2016; Published November 15, 2016

Abstract: Metastasis represents the main cause of cancer-related death. A minority of patients were firstly diagnosed with solid tumor of unknown origin in bone marrow (BM-STUOs) before the appearance of clinical symptoms. We carried out an eight-year follow-up study for 94 patients (66 men and 28 women) with BM-STUOs. The median age of patients enrolled in this research was 58 years. Most of them (79, 84.0%) complained dizziness or fatigue. Fifty-four patients (57.4%) were admitted to their neighborhood hospital with local or widespread bone pain. Anemia (29.8%), thrombocytopenia (13.5%) and pancytopenia (9.0%) were the most common laboratory findings. To gain more insights into the lesions, bone marrow biopsy was undertaken. Histologically, malignant cells were arranged in clusters and surrounded by fibroblasts. Osteogenic and osteoclastic reactions were obvious. However, the primary sites of these metastatic lesions are always obscure because of the lack of histological evidence. A panel of immunohistochemical antibodies was employed to identify the phenotype of tumor cells. Unfortunately, definite diagnosis was established in only 48 patients. Kaplan-Meier analysis indicated that the prognosis of patients with definite origin was better than the patients without ($P<0.05$). Furthermore, the time consumption of diagnosis will affect the survival of patients with BM-STUOs.

Keywords: Bone marrow, metastasis, biopsy, cancer, immunohistochemistry

Introduction

During the last decade, rapid improvement of modern medical techniques promotes the flourish of diagnosis and therapeutic strategies, which bring a more favorable prognosis to cancer patients than ever before [1-3]. Nevertheless, many studies have appeared in the literature from different parts of the world on bone marrow (BM) invasion by solid tumors, accounting for the vast majority of cancer-related deaths worldwide and representing both a diagnostic and a management challenge [4].

It has been widely accepted that the occult haematogenous metastasis of operable cancer could occur in very early stage. Although the underlying pathogenic mechanisms remain unknown, most of the tumor cells, successfully escaping the primary site, enter peripheral blood (PB) and subsequently disseminate to secondary organs such as BM. From a clinical perspective, if the metastatic cascade could be halted in this stage, good prognosis of such patients could be expected. However, before effectively employing systemic cancer treatment for occult metastatic cells, the identity of primary tumors needs to be clarified. BM plays a prominent role as an indicator organ of occult tumor cell dissemination because it is easily accessible by aspiration and repeated BM aspiration is associated with extremely low patient morbidity.

In present study, we focused on patients with solid tumors of unknown origin in bone marrow

Table 1. Summary of Primary Antibodies Used for Immunohistochemistry

Antibody	Clone	Isotype	Supplier	Dilution
Cytokeratin	AE1/AE3	IgG1 (mouse)	DAKO	1:50
E-Cadherin	NCH-38	IgG1 (mouse)	DAKO	1:100
GCDFP-15	23A3	IgG2a (mouse)	Novocastra	1:50
PSA	28A4	IgG1 (mouse)	Novocastra	1:100
PSMA	1D6	IgG1 (mouse)	Novocastra	1:50
PAP	PASE/4LJ	IgG1 (mouse)	Novocastra	1:100
Tyroglobulin	1D4	IgG2a (mouse)	Novocastra	1:100
SP-A	PE10	IgG2b (mouse)	DAKO	1:50
TTF-1	8G7G3/1	IgG1 (mouse)	DAKO	1:150
P63	4A4	IgG2a (mouse)	DAKO	1:50
Cytokeratin 7	OV-TL 12/30	IgG1 (mouse)	DAKO	1:100
Cytokeratin 20	Ks20.8	IgG2a (mouse)	DAKO	1:50
CEA	II-7	IgG1 (mouse)	DAKO	1:300
CDX2	AMT28	IgG1 (mouse)	Novocastra	1:100
Hep Par1	OCH1E5	IgG1 (mouse)	DAKO	1:50
AFP	C3	IgG2a (mouse)	Novocastra	1:50
HBsAg	3E7	IgG1 (mouse)	DAKO	1:100

(BM-STUOs). A BM-STUO is defined as a metastatic cancer in bone marrow firstly confirmed by BM biopsy, the primary tumor of which is not apparent, and the origin may or may not be found at post examinations [5]. Because of sharing many histological characteristics with each other, BM-STUOs of different origins sometimes are hard to be distinguished under light microscope. Taking advantage of immunohistochemical (IHC) staining, we could obtain potent evidence for the cellular hallmarks of the primary tumors. However, the available data for comprehensive immunophenotypic analysis of BM-STUOs is limited, even though there is accumulating studies about metastatic diseases involving BM. So, we analyzed samples by using conventional HE and immunohistochemistry (IHC) to contrast the histopathological and molecular differences among BM-STUOs of different origins. In order to gain more insights into the determinants of survival, we further evaluated the effect of different factors on the prognosis of these patients.

Materials and methods

Included in this study were all cases of BM-STUOs, diagnosed by bone marrow biopsy between February 2009 and February 2016. Ninety-four patients were enrolled with a median follow-up of 43 months and gave informed consent to the study. The study was approved by Nanchang University Research Ethics Committee.

The data of each case, including clinical features, endoscopic results, cytology, imaging and serological tests etc., were recorded in full. The final diagnosis of primary tumor was based on the aggregate analysis of histopathological features, immunophenotype and other examinations. The final diagnosis interval was defined as a time span between first BM biopsy and the final diagnosis of primary tumor. All patients were followed until April 30 2016.

Immunohistochemical staining

All biopsy specimens decalcified in EDTA were fixed in 10% neutral-buffered formalin and embedded in paraffin for histological and immunohistochemical analysis [6]. The deparaffinised tissue sections were bathed in 0.01 mol/L citrate buffer (pH 6.0) to retrieve the antigens at 95°C~99°C for 10 minutes followed by peroxidase blocking in 3% hydrogen peroxide solution and incubation with mouse primary antibody overnight at 4°C (**Table 1**) and HRP-conjugated secondary antibody (30 minutes at room temperature, DAKO EnVision™, Glostrup, Denmark). The staining signals were visualized using DAB.

Statistical analysis

Kaplan-Meier survival curves were generated and the log-rank test was employed to compare them. Stepwise multivariate Cox regression analysis was used to calculate corrected hazard ratios and 95% confidence intervals (CI) for cumulative survival. In all statistical analyses, $P<0.05$ was considered significant. All calculations were performed using the SPSS 13.0 statistical package (SPSS, Inc., Chicago, IL).

Results

Clinical features and follow-up data analysis

The study is ongoing, but April 30, 2016, was the cutoff date for the present study. A total of

Table 2. The results of main serological tests of patients with BM-STUOs

Case	Gender	Age (years)	Primary Tumor	Erythrocyte ($\times10^9$/l)	Leucocyte ($\times10^9$/l)	Platelet ($\times10^9$/l)	Hemoglobin (g/l)	Calcium (mmol/l)	AFP (ng/ml)	PSA (ng/ml)
1	F	65	Stomach-poorly differentiated adenocarcinoma	2.23	7.25	187	55	2.53	2.05	-
2	M	32	Stomach-signet ring cell carcinoma	3.02	4.91	249	74	2.42	4.01	0.91
3	M	44	Stomach-signet ring cell carcinoma	1.59	6.82	129	72	2.32	1.03	1.03
4	F	50	Stomach-signet ring cell carcinoma	2.39	2.77	87	101	4.67	2.21	-
5	M	37	Hepatocellular carcinoma	4.21	6.21	201	141	2.61	647.70	0.43
6	M	48	Hepatocellular carcinoma	1.98	6.23	281	49	2.67	3.04	2.01
7	M	51	Hepatocellular carcinoma	1.86	7.21	218	81	2.41	543.36	0.49
8	M	52	Hepatocellular carcinoma	4.54	5.98	148	136	2.49	1111.46	1.94
9	M	55	Hepatocellular carcinoma	2.71	6.88	291	107	2.38	5845.00	3.00
10	F	57	Hepatocellular carcinoma	2.35	8.21	193	79	2.39	173.47	-
11	M	64	Hepatocellular carcinoma	4.77	7.81	195	125	2.42	713.40	1.71
12	M	68	Hepatocellular carcinoma	3.71	3.22	79	83	2.39	421.10	0.83
13	F	75	Hepatocellular carcinoma	3.02	5.28	173	58	2.39	65.06	-
14	M	77	Hepatocellular carcinoma	4.89	7.32	174	130	2.31	54.27	1.94
15	M	50	Lung-squamous cell carcinoma	4.90	7.32	130	138	2.38	2.31	3.71
16	M	68	Lung-squamous cell carcinoma	5.11	6.43	282	127	2.18	3.01	2.18
17	F	48	Lung-adenocarcinoma	3.01	6.21	273	78	2.67	1.23	–
18	F	52	Lung-adenocarcinoma	4.32	5.03	134	125	2.40	0.45	-
19	M	57	Lung-adenocarcinoma	4.87	8.22	240	131	2.48	1.93	0.81
20	M	57	Lung-adenocarcinoma	3.12	2.08	65	70	5.81	2.01	1.34
21	F	61	Lung-adenocarcinoma	3.69	5.61	201	127	2.50	3.00	-
22	M	61	Lung-adenocarcinoma	2.46	2.14	87	91	9.33	2.12	2.71
23	F	62	Lung-adenocarcinoma	3.87	4.98	231	119	2.58	2.90	-
24	M	64	Lung-adenocarcinoma	5.01	8.14	210	130	2.31	1.92	2.04
25	M	72	Lung-adenocarcinoma	2.47	6.98	212	67	2.51	2.10	1.55
26	M	72	Lung-adenocarcinoma	5.23	5.61	238	138	2.41	0.07	3.01
27	M	56	Thyroid gland-follicular carcinoma	4.98	7.33	250	140	2.59	0.21	3.32
28	M	43	Colon-adenocarcinoma	5.21	8.41	177	148	2.37	4.12	0.46
29	M	46	Colon-adenocarcinoma	4.76	7.30	198	137	2.70	5.02	0.79
30	M	64	Prostate-adenocarcinoma	3.01	3.87	84	91	2.18	3.33	64.03
31	M	65	Prostate-adenocarcinoma	4.69	5.14	205	129	2.51	2.81	52.87
32	M	67	Prostate-adenocarcinoma	4.91	6.34	230	126	2.66	1.31	71.22
33	M	73	Prostate-adenocarcinoma	1.77	4.02	137	65	2.54	1.90	40.49
34	M	74	Prostate-adenocarcinoma	5.03	8.10	216	139	2.19	2.01	66.81
35	F	31	Breast-invasive ductal carcinoma	4.00	6.99	261	131	2.52	3.81	-

36	F	35	Breast-invasive ductal carcinoma	2.67	6.43	249	82	2.37	2.01	-
37	F	48	Breast-invasive ductal carcinoma	4.02	7.31	183	128	2.49	1.11	-
38	F	49	Breast-invasive ductal carcinoma	2.11	5.29	223	63	2.16	1.02	-
39	F	54	Breast-invasive ductal carcinoma	4.12	6.81	188	120	2.62	3.11	-
40	F	55	Breast-invasive ductal carcinoma	3.88	7.14	159	119	2.39	3.94	-
41	F	56	Breast-invasive ductal carcinoma	3.91	9.31	209	125	2.38	2.39	-
42	F	58	Breast-invasive ductal carcinoma	2.13	1.86	57	82	4.32	3.85	-
43	F	58	Breast-invasive ductal carcinoma	4.23	5.06	215	130	2.13	4.12	-
44	F	62	Breast-invasive ductal carcinoma	2.59	9.12	184	97	2.35	0.34	-
45	F	88	Breast-invasive ductal carcinoma	3.71	6.71	172	125	2.48	0.55	-
46	M	73	Esophagus-squamous cell carcinoma	5.12	4.78	210	133	2.40	0.57	0.32
47	M	75	Esophagus-squamous cell carcinoma	4.73	5.30	220	132	2.53	1.02	0.02
48	M	74	Esophagus-adenocarcinoma	4.98	6.81	219	150	2.69	2.00	2.01
49	M	27	Undetermined	5.12	5.98	284	146	2.51	0.58	0.01
50	M	29	Undetermined	2.98	7.45	281	58	2.60	2.81	1.09
51	M	35	Undetermined	5.25	6.01	240	125	2.59	3.01	1.59
52	M	37	Undetermined	4.99	4.98	230	127	2.64	1.03	1.9
53	F	42	Undetermined	3.60	5.39	258	122	2.39	0.09	-
54	M	44	Undetermined	2.97	9.33	193	66	2.38	0.93	0.69
55	M	44	Undetermined	2.67	2.90	65	78	2.19	0.81	1.92
56	F	48	Undetermined	2.96	1.83	90	90	2.43	2.89	-
57	F	48	Undetermined	3.88	8.05	179	132	2.32	0.71	-
58	M	48	Undetermined	3.08	5.73	166	93	2.51	4.01	2.14
59	M	48	Undetermined	4.85	7.31	240	135	2.61	4.44	1.31
60	M	48	Undetermined	3.18	2.71	76	69	2.20	1.09	2.04
61	M	49	Undetermined	4.01	5.92	258	133	2.43	3.01	2.85
62	M	49	Undetermined	3.11	6.70	198	58	2.48	1.90	0.82
63	M	49	Undetermined	4.39	7.50	280	130	2.49	2.59	1.32
64	F	50	Undetermined	3.97	7.31	160	117	2.24	3.01	-
65	M	50	Undetermined	2.80	8.21	251	57	2.39	2.83	2.95
66	M	51	Undetermined	4.82	6.51	158	125	2.39	3.84	3.01
67	M	52	Undetermined	4.30	4.81	169	135	2.28	0.06	0.82
68	M	52	Undetermined	2.39	5.12	164	82	2.43	0.91	3.41
69	F	53	Undetermined	4.02	4.59	158	130	2.51	1.06	-
70	M	54	Undetermined	4.71	6.81	157	140	2.43	3.90	2.01
71	M	58	Undetermined	3.21	5.29	166	94	2.64	0.04	1.99
72	M	59	Undetermined	4.33	5.71	190	127	2.61	2.31	1.74
73	M	60	Undetermined	2.91	5.62	188	89	2.19	4.09	2.74

74	M	62	Undetermined	4.95	4.90	284	149	2.14	2.41	1.56
75	M	62	Undetermined	3.33	7.37	209	99	2.41	0.51	0.04
76	F	64	Undetermined	4.71	8.31	200	125	7.21	0.71	-
77	F	64	Undetermined	4.82	7.31	185	126	2.38	4.31	-
78	M	64	Undetermined	2.72	5.28	281	62	2.57	5.00	0.41
79	M	65	Undetermined	4.79	4.68	175	141	2.41	1.03	1.90
80	M	65	Undetermined	3.71	5.39	173	87	2.55	4.00	2.19
81	F	67	Undetermined	1.35	1.67	49	63	2.11	1.23	-
82	F	68	Undetermined	3.90	6.31	175	121	2.59	1.98	-
83	M	69	Undetermined	4.63	6.32	220	132	2.63	5.31	3.01
84	M	71	Undetermined	3.29	6.81	248	84	2.59	2.01	1.24
85	M	73	Undetermined	4.77	5.10	165	125	2.33	3.08	2.91
86	M	73	Undetermined	3.55	9.25	206	102	2.14	6.88	3.04
87	M	74	Undetermined	4.61	4.37	249	135	2.58	3.56	2.54
88	M	74	Undetermined	5.31	5.30	260	121	2.62	3.59	2.39
89	M	74	Undetermined	5.08	6.31	271	136	2.37	3.01	1.92
90	M	75	Undetermined	4.89	5.98	189	142	2.19	2.56	0.84
91	M	76	Undetermined	4.75	6.90	205	129	2.38	1.72	1.13
92	M	78	Undetermined	5.12	7.38	179	128	2.38	1.85	3.11
93	M	79	Undetermined	3.19	8.33	183	108	2.23	3.00	2.46
94	M	85	Undetermined	4.83	5.09	230	130	2.67	4.91	2.17

Table 3. Univariate analysis of clinicopathological factors

Characteristic	No. of patients	*P* value*
Age		0.420
<30 yr	2	
30-39 yr	6	
40-49 yr	18	
50-59 yr	24	
60-69 yr	23	
≥70 yr	21	
Gender		0.035
Male	66	
Female	28	
Primary site		0.592
Esophagus	3	
Stomach	4	
Colon	2	
Liver	10	
Lung	12	
Thyroid gland	1	
Prostate	5	
Breast	11	
Undetermined	46	
Diagnosis of primary tumor		0.001
Determined	48	
Undetermined	46	
Final Diagnosis Interval		0.001
≥1 month	28	
1-2 months	12	
>2 months	8	

**P* values were calculated with the use of the log-rank test.

94 patients (66 male and 28 female), whose age ranged from 27 to 88 years (median, 58 years), was diagnosed with BM-STUOs, and the follow-up period for these patients ranged from 8 to 89 months, with a median of 43 months. However, the median survival was a mere 11 months.

The most common symptoms of these patients were local or systemic bone pain (33, 35.1%) and persistence low grade fever (13, 13.8%). Three patients displayed hepatomegaly or splenomegaly. Anemia (28, 29.8%), thrombocytopenia (15, 16.0%), pancytopenia (10, 10.6%) and hypercalcemia (5/94, 5.3%) were the most common hematological findings (**Table 2**). A proportion of patients received preliminary consideration of hematopoietic system diseases before the pathological diagnosis of BM biopsy is granted.

Although conventional histopathological and immunohistochemical examination of BM biopsy sections, combined with imaging and PB examinations, could indicate the primary site of BM-STUOs, the final diagnosis of primary tumor was based on the pathological results of surgical resection, fine needle aspiration biopsy or endoscope. Forty-eight patients (48/94, 51.1%) received final diagnosis of primary tumor (**Table 2**). Seven of 10 cases with HCC had high level serum HBsAg, and 9 patients showed high level of serum AFP. Abnormal serum PSA and F-PSA were detected in all of 5 patients with prostate carcinoma (**Table 2**).

Univariate analysis (using the log-rank test or the Cox model) showed that age (*P*=0.420) and histological type of primary tumor (*P*=0.592) bear no significant correlation with prognosis (**Table 3**). On the other hand, three significant prognostic factors were identified: first, female patients were related with longer survival (*P*=0.035); second, the prognosis of patients whose primary tumor was confirmed was better than those who did not (*P*=0.001); and third, the final diagnosis interval could contribute to the survival rate (*P*=0.001). BM-STUOs patients, who received final diagnosis of primary site within one or two months and subsequent employed tailored systemic treatment, had longer survival time than those over 2 months (both *P*=0.001). But there was no significant difference between the first two groups (*P*=0.387) (**Figure 1**). The final diagnosis interval and definite diagnosis of primary site remained independently associated with cumulative survival of patients with BM-STUOs after Multivariate Cox regression analysis (**Table 4**).

Histological and immunophenotypic characteristics of BM-STUOs

Breast: In adult females, adenocarcinoma of the breast is the most common metastatic tumor in the bone marrow [7]. The histological type of 11 patients with breast cancer was invasive ductal carcinoma in which the youngest was 31 years old. Imaging examinations found five cases of abnormal bone changes of which two cases had systemic bone lesions. The main destructions observed in imaging pic-

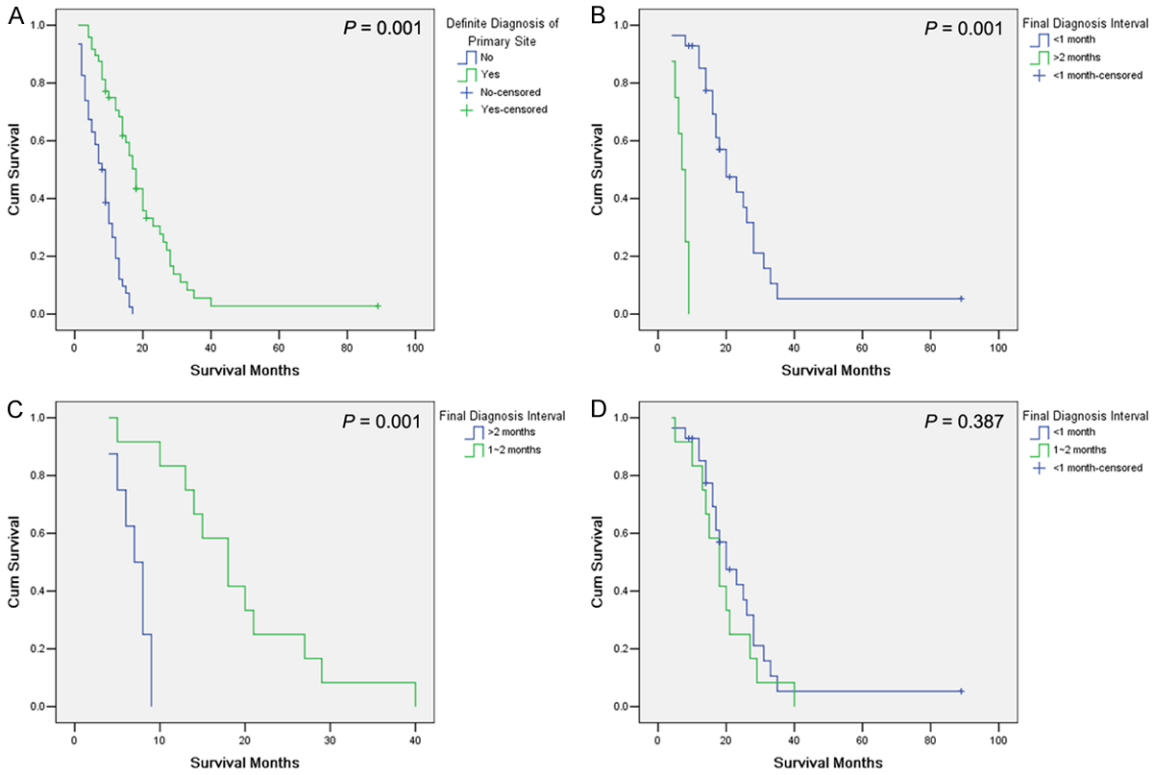

Figure 1. Cumulative survival curves of patients with BM-STUOs. Among 94 cases, patients received definite diagnosis of primary site had different cumulative survival curves to those with unclear diagnosis (A). If the final diagnosis could be confirmed within 1 month or 1~2 months, patients would have better prognosis than those over 2 months (B and C). However, cumulative survival curves showed no significantly difference between patients with final diagnosis confirmed within 1 month and 1~2 months (D).

Table 4. Multivariant analysis of gender, definite diagnosis of primary site and final diagnosis interval

		95.0% CI for Exp (B)		
	Exp (B)	Lower	Upper	Sig.
Gender				0.283
Definite diagnosis of primary site	4.919	2.865	8.447	0.000
Final Diagnosis Interval	2.729	1.629	4.572	0.000

tures were moth-eaten like or patchy. In most cases of bone marrow biopsy, osteolysis and bone remodeling were present, and an equal number of cases exhibited desmoplastic reaction. Tumor cells assembled as nest or abortive duct, and characterized apocrine gland metaplasia could be seen in some ducts with hobnail-like epithelial cells [8-10]. Malignant cells can be large with abundant and eosinophilic cytoplasm and prominent nucleolus. All cases expressed AE1/AE3 and E-Cadherin, and eight patients were also positive for GCDFP-15 (**Figure 2**).

Prostate

Carcinomas of prostate gland show a tendency to involve bone marrow which is not associated with the Gleason grade of primary tumor [11]. Extensive osteolytic lesions could be seen in bone imaging of most patients who were usually diagnosed with multiple myeloma. Desmoplastic reactions were prominent, though the degree was variable through the specimen (**Figure 3**). Tumor cells infiltrated normal bone marrow tissue with patterns of solid, clear cell or cord. Some cases displayed neuroendocrine features. Strikingly enlarged and eosinophilic nucleoli were visible in the nuclei of cancer cells in asymmetrical or central position [12]. PSA exclusively expressing in BM metastatic diseases of prostate origin facilitated the diagnosis (**Figure 3**). Combined with positive staining for PSMA, PAP and the level of PSA and PSMA in PB, the histological origin could be almost confirmed.

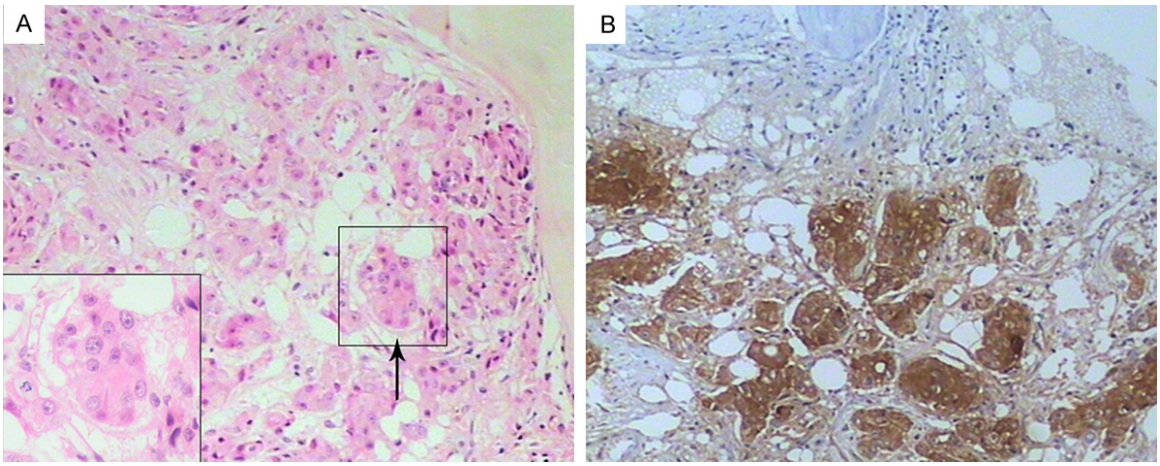

Figure 2. Invasive ductal carcinoma of breast. A. Tumor cells were arranged in clusters and trabeculae (H&E, original magnification ×200; inset, original magnification ×400). B. Clusters of tumor cells express GCDFP-15 (GCDFP-15, original magnification ×200).

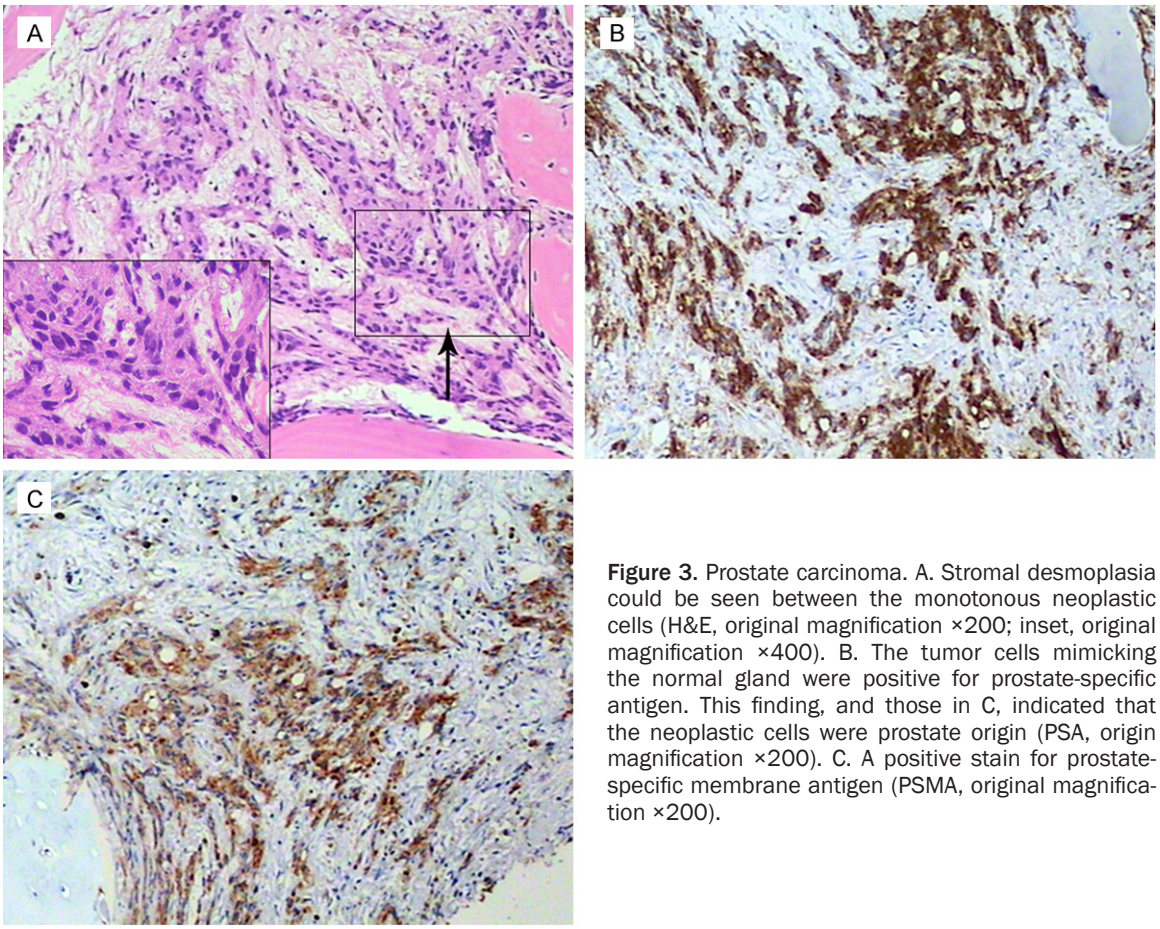

Figure 3. Prostate carcinoma. A. Stromal desmoplasia could be seen between the monotonous neoplastic cells (H&E, original magnification ×200; inset, original magnification ×400). B. The tumor cells mimicking the normal gland were positive for prostate-specific antigen. This finding, and those in C, indicated that the neoplastic cells were prostate origin (PSA, origin magnification ×200). C. A positive stain for prostate-specific membrane antigen (PSMA, original magnification ×200).

Lung

Carcinomas of lung were the most common type in 48 diagnosed patients, 10 patients of which were adenocarcinoma and 2 cases were squamous cell carcinoma. The gender ratio was 2:1 (8 male and 4 female), and all four female patients were adenocarcinoma. Both

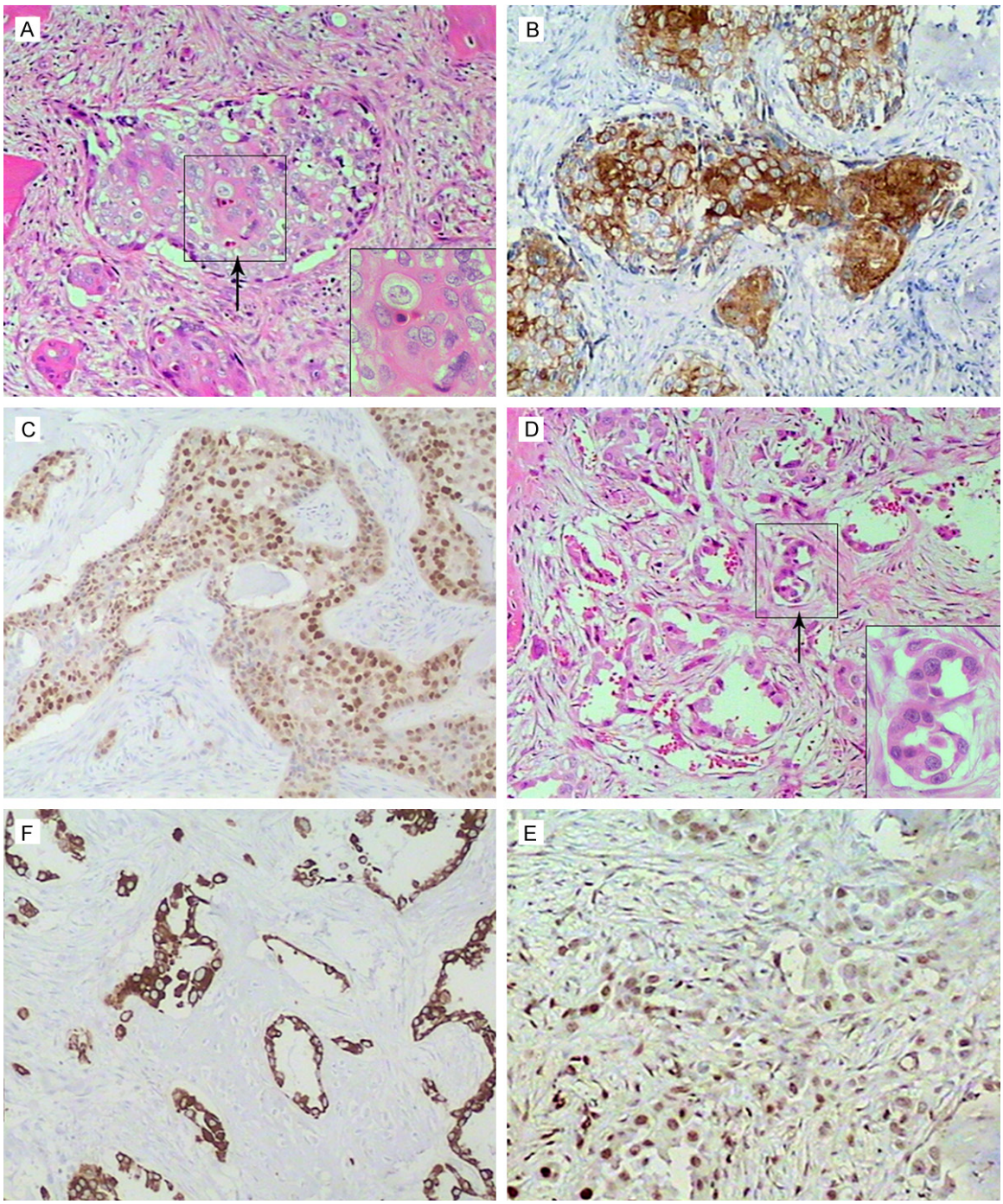

Figure 4. Lung carcinoma. (A-C) Trabeculae disorganization and fibrosis could be seen in most of squamous cell carcinomas. The expression of 34βE12 showed sharp demarcation from the fibrosis stroma and squamous pearl formation. Nucleus were stained by P63; H&E, original magnification ×200 (A); inset, original magnification ×400; 34βE12, original magnification ×200 (B); P63, original magnification ×200 (C). (D-F) In adenocarcinoma, the signs of bone resorption and stromal desmoplasia surrounded irregular carcinomatous nests which were lined by atypical cuboidal cells were common. Tumor cells expressed both TTF-1 and AE1/AE3; H&E, original magnification ×200 (D); inset, original magnification ×400; TTF-1, original magnification ×200 (E) and AE1/AE3, original magnification ×200 (F).

local and systemic bone lesions could be observed in imaging pictures. One of the most common characteristics of adenocarcinoma was the extensive osteolytic lesions combined

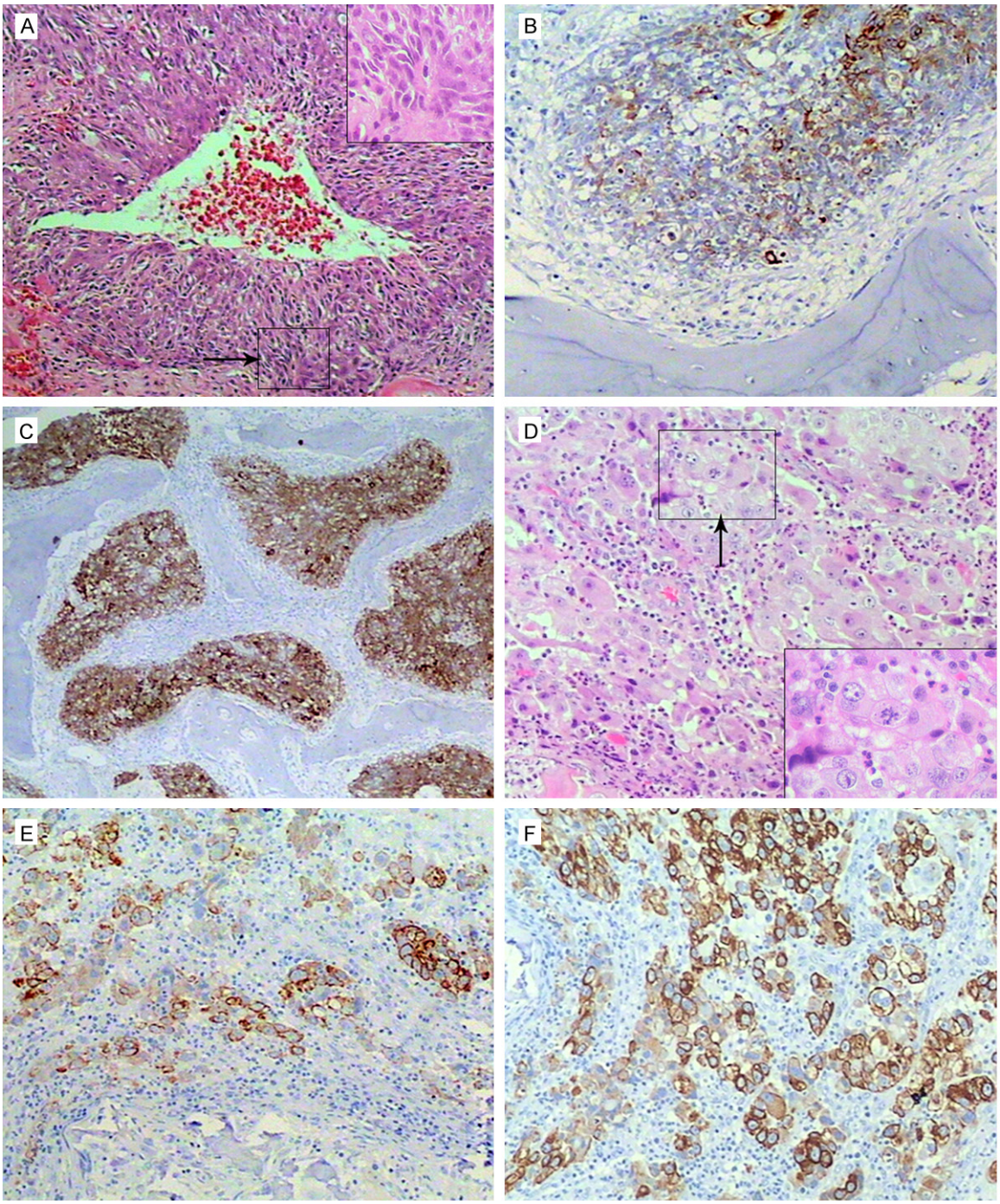

Figure 5. Esophagus carcinoma. (A-C) Squamous carcinoma showed a discrete cell nest distinct from the fibrous stroma and there was central necrosis; Tumor nests were positive for 34βE12 and CK7; H&E, origin magnification ×200 (A); inset, original magnification ×400; 34βE12, origin magnification ×200 (B) and CK7, origin magnification ×200 (C). (D-F) Adenocarcinoma was consisted with poorly formed tubules diffusely infiltrating the bone marrow, the stain patterns of neoplastic cells were positive for CK7 and AE1/AE3; H&E, origin magnification ×200 (D); inset, original magnification ×400; CK7, origin magnification ×200 (E) and AE1/AE3, origin magnification ×200 (F).

with fibrosis (**Figure 4**). Tumor cells, with abundant cytoplasm and distinct nucleoli, were arranged in abortive alveolus as a para-trabeculae pattern. In patients with metastatic squamous cell carcinomas, cell nests were embedded in prominent fibrosis which induced destruction and remodeling of bone trabeculae (**Figure 4**). Poorly differentiated cells clearly

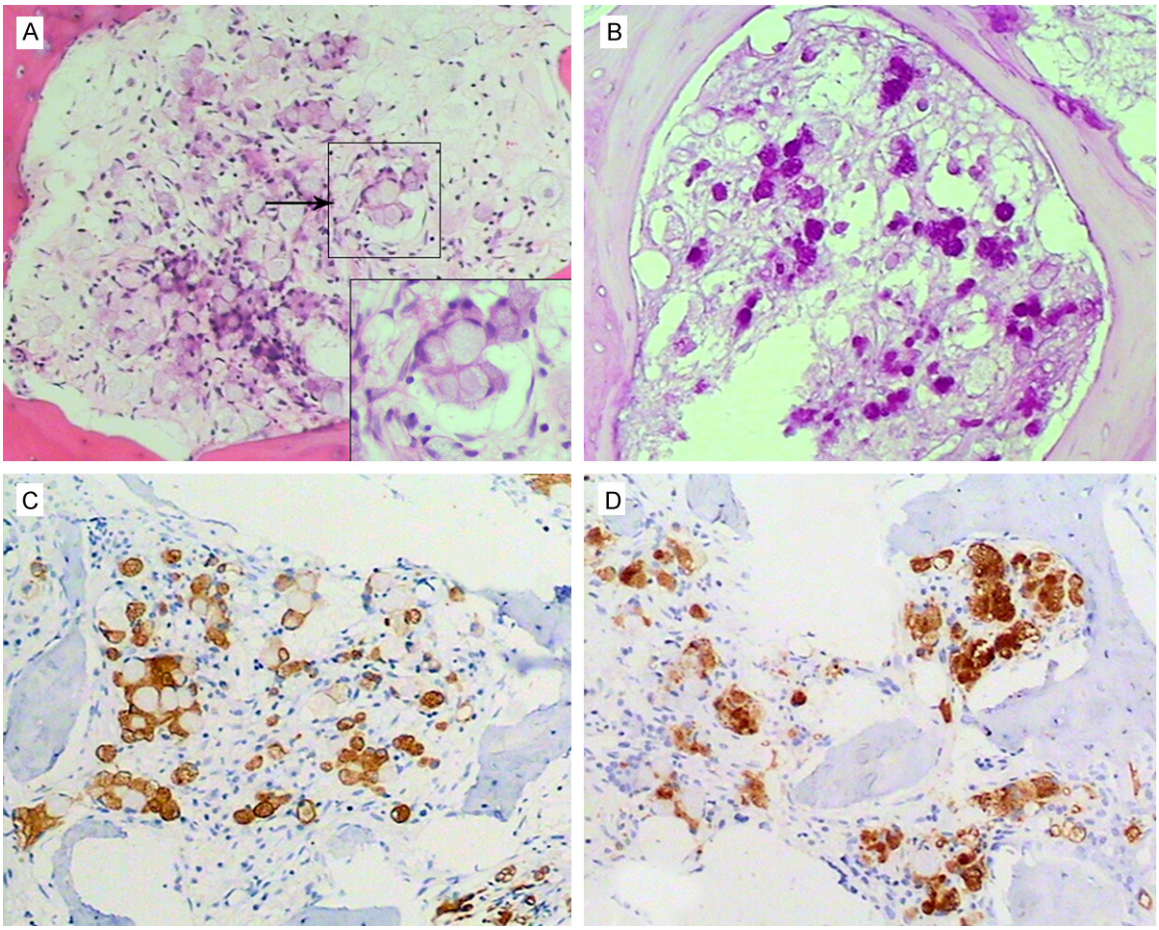

Figure 6. Stomach signet-ring cell carcinoma. (A) Cluster of signet-ring cells (H&E, origin magnification ×200; inset, original magnification ×400). (B) Tumor cells were positive in mucin stain pattern (PAS, origin magnification ×200). (C and D) Stains for CK7 and CEA decorated the scattered ring cells; CK7, origin magnification ×200 (C) and CEA, origin magnification ×200 (D).

showed keratinization [13]. Amorphous necrotic debris and pathologic keratin pearl could be observed in some nests. All cases of adenocarcinoma and squamous cell carcinoma were negative for TG and surfactant apoprotein A (PE-10). Adenocarcinoma cells expressed TTF-1, AE1/AE3 and CK7 (**Figure 4**). Dramatically, both of 2 cases of squamous showed negative staining pattern of TTF-1, although they were positive for 34βE12.

Digestive system

Compared with patients with breast, prostate or lung cancers, the frequency that metastatic tumor of gastrointestinal tract involves BM is lower [14]. Three cases of BM-STUOs were confirmed with origin in esophagus, two of which were squamous cell carcinoma and the other one was adenocarcinoma. Most areas of squamous cell tumor were composed of basoloid cells arranging in trabecular or solid patterns [15], but there were large differentiated squamous cells containing large vesicular nuclei and prominent nucleoli scattered throughout (**Figure 5**). Necrotic debris also could be seen in some areas. Monoclonal antibody against 34βE12 may help to distinguish it from poorly differentiated adenocarcinoma (**Figure 5**). Although abnormal bone tissues were rare, desmoplastic reaction and relict trabeculae could always be observed in the biopsy specimens of adenocarcinoma. Some abortive gland scattered in diffused tumor tissues which showed accumulation of inflammation cells [16]. Malignant cells with prominent nuclei and nucleoli displayed significant atypia and mitotic figure. The immunoperoxidase staining pattern was CK7 (+) and CK20 (-) which was similar to adenocarcinoma originating in stomach (**Figure 5**).

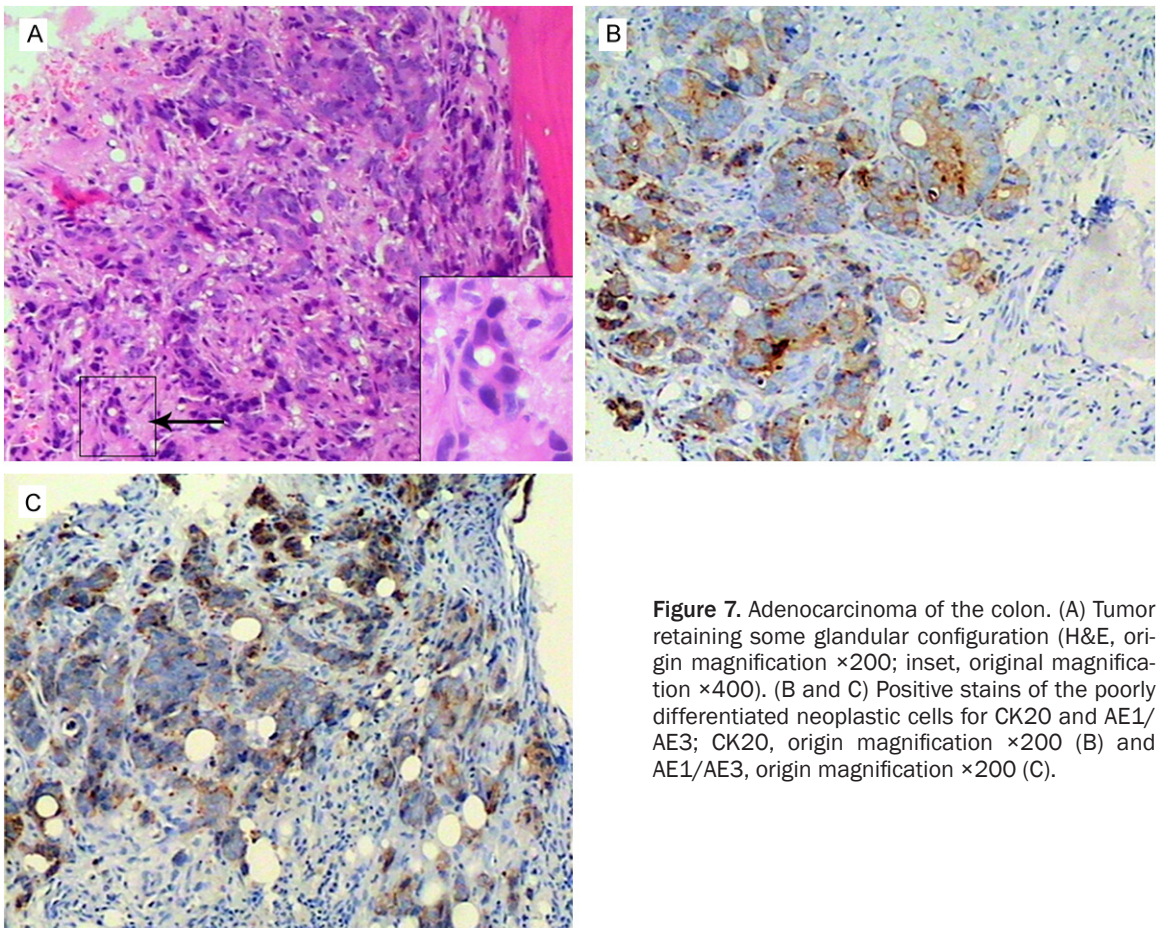

Figure 7. Adenocarcinoma of the colon. (A) Tumor retaining some glandular configuration (H&E, origin magnification ×200; inset, original magnification ×400). (B and C) Positive stains of the poorly differentiated neoplastic cells for CK20 and AE1/AE3; CK20, origin magnification ×200 (B) and AE1/AE3, origin magnification ×200 (C).

In the present study, three of four patients diagnosed with stomach carcinoma were signet cell carcinoma, which is the most frequent subtype of stomach tumor involving bone marrow, and the other one was poorly differentiated carcinoma. PAS staining showed that a number of densely purple staining signet cells arranged in nests or individually distributed (**Figure 6**). Signet cells expressed CK7 rather than CK20 and this immunophenotypic pattern can be used in the differential diagnosis against colon adenocarcinoma, because both of them were positive for CEA (**Figure 6**). Prominent desmoplastic reaction and "dirty" abortive gland existed in most cases of colon adenocarcinoma (**Figure 7**). The cancer cells were positive for AE1/AE3, CEA and CK20, and CK7 staining was negative. CDX-2, indicating gastrointestinal origin, was another useful antibody [17, 18].

Ten patients (8 male and 2 female) were diagnosed with metastatic HCC. The tumor cells grew in thickened cords, mimicking the cell plates of normal liver (**Figure 8**). The trabeculae were lined by epithelial cells, and the sinusoidal-like spaces could be seen between them. Some tumor cells contained bile [19, 20]. Immunohistochemical staining showed that all cases were positive for Hep Par1, and 5 of 7 cases who had high level serum HBsAg were also expressed HBsAg. Nine patients were reported with both high level of serum AFP and positive staining for AFP (**Figure 8**).

Thyroid gland

Bone marrow is the most frequently involved secondary organ by metastatic thyroid tumors [21]. Although follicular carcinoma is relatively rare, it appears to possess a more aggressive ability compared with papillary carcinoma. The normal bone marrow tissues were diffusely replaced by tumor cells. Some areas were solid or microfollicular. There was abundant thyroglobulin-like eosinophilic matrix in the central sinus of abortive alveolus [22, 23]. Tumor cells showed light cytological atypia. TTF-1 was useful in identifying thyroid origin of BM-STUOs, as are HBME-1 and TG (**Figure 9**).

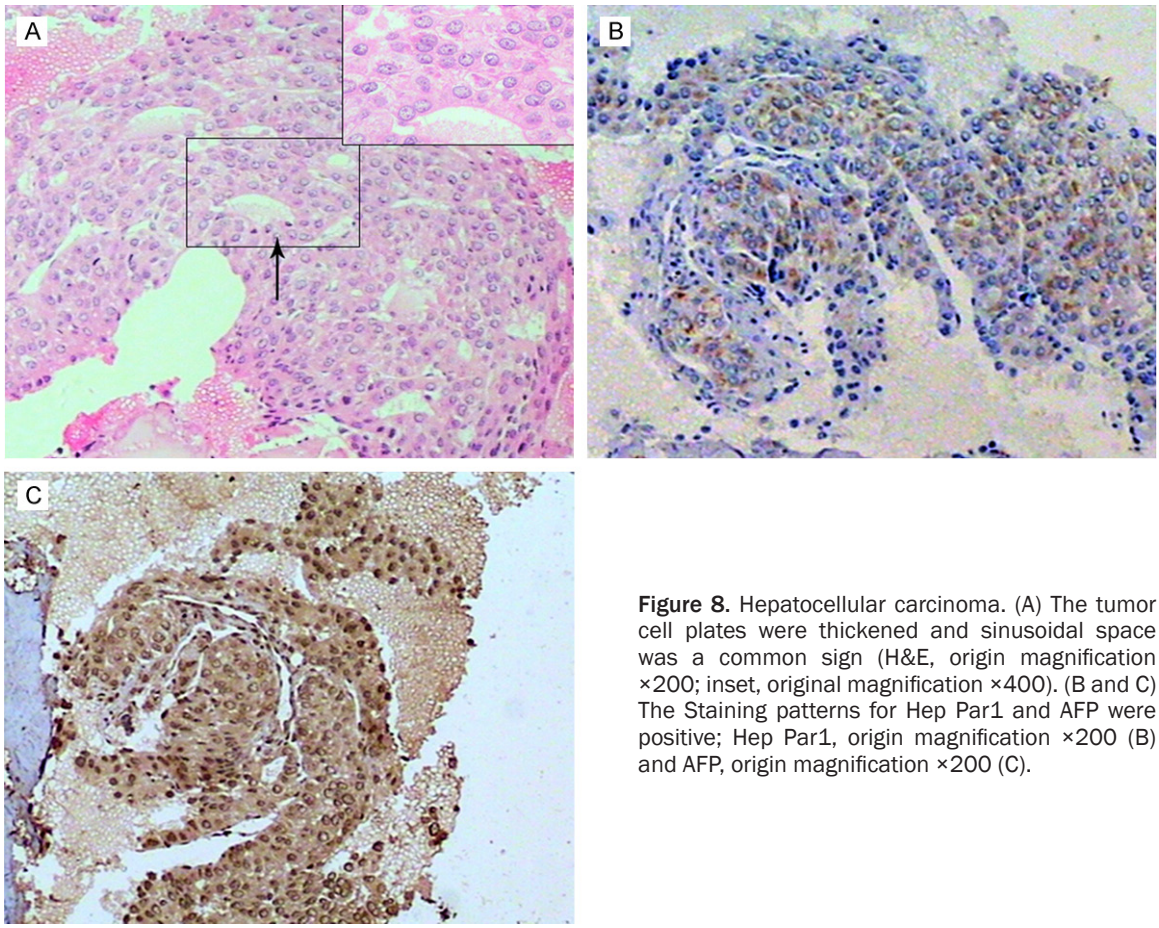

Figure 8. Hepatocellular carcinoma. (A) The tumor cell plates were thickened and sinusoidal space was a common sign (H&E, origin magnification ×200; inset, original magnification ×400). (B and C) The Staining patterns for Hep Par1 and AFP were positive; Hep Par1, origin magnification ×200 (B) and AFP, origin magnification ×200 (C).

Discussion

One of the major obstacles in battle against cancer is the presence of recurrence. According to previous studies, occult metastasis contributes mainly to the lethal destruction of secondary organs [24, 25]. However, most of the patients with occult metastasis received insufficient attention because the symptoms related to BM metastasis are variable and nonspecific, and which, in turn, hampered the employment of systemic treatment. Consistent with published literature, our results indicate that a proportion of patients with BM-STUOs were primarily considered as hematopoietic diseases because most of them displayed anemia and/or bone pain. If the primary tumor of patients with BM-STUOs could be determined in a short time (less than 2 months), when metastatic diseases burden was low and amenable to several interventions, including, but not limited to, potentially curative surgical resection of all visible tumor and chemotherapy, better prognosis could be expected.

However, the purpose of locating the exact position of primary tumor immediately could be hardly achieved by using imaging and conventional histopathological techniques, because the metastatic lesions share many pathological features and the primary tumors are sometimes too small to be detected. The development of monoclonal antibodies against tumor-associated cell proteins has opened a new diagnostic window, even allowing the identification of individual disseminated cancer cell against 10^6 to 10^7 normal BM cells. In the present study, we firstly provided insights into the comprehensive analysis of BM biopsy specimens obtained from patients with BM-STUOs. Our results showed that there were many differences among different types of poorly differentiated carcinomas, which constituted the major proportion of BM-STUOs. Cells in breast invasive ductal carcinoma had abundant cytoplasm, prominent nucleoli, and arranged in nests or abortive ducts. Prostate carcinoma tissue displayed cords pattern on a background of fibrosis, and clear cells could be observed in

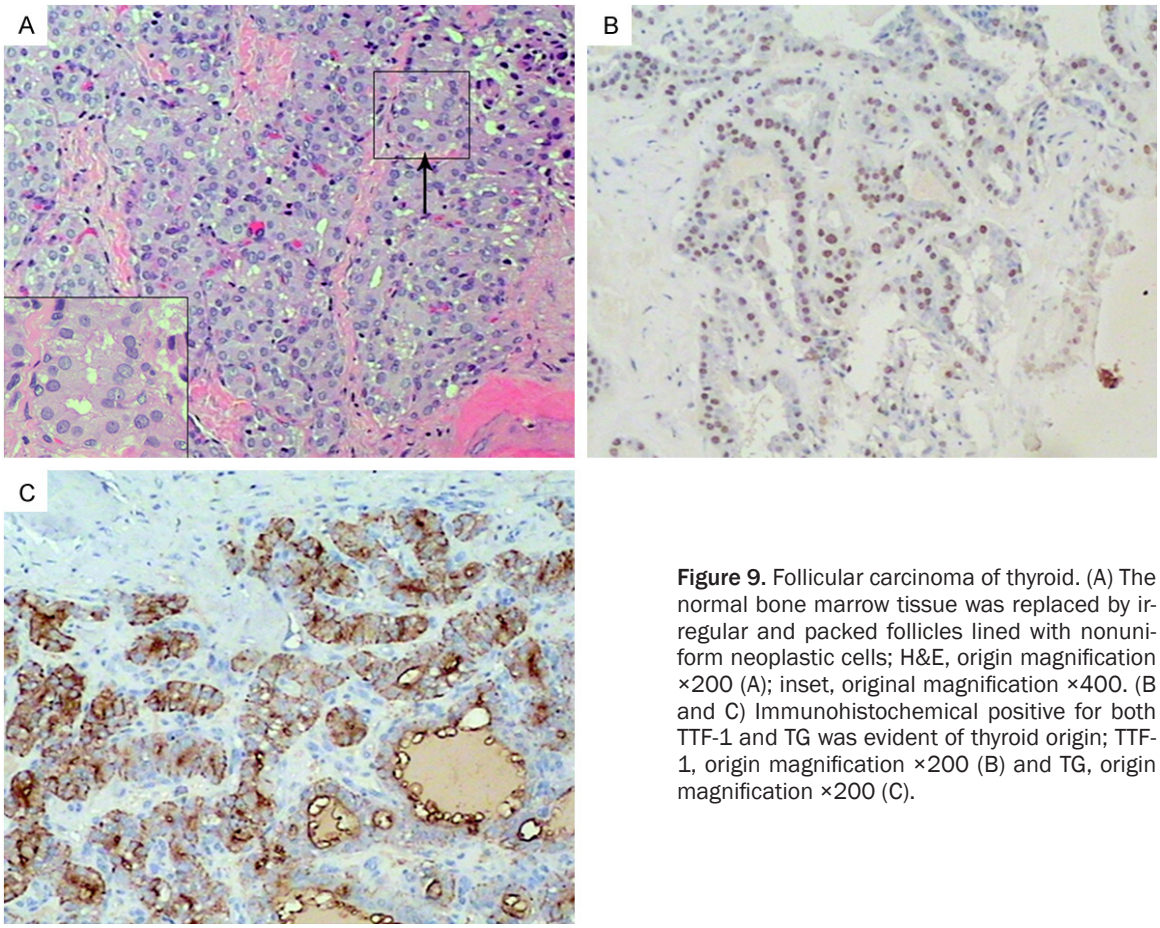

Figure 9. Follicular carcinoma of thyroid. (A) The normal bone marrow tissue was replaced by irregular and packed follicles lined with nonuniform neoplastic cells; H&E, origin magnification ×200 (A); inset, original magnification ×400. (B and C) Immunohistochemical positive for both TTF-1 and TG was evident of thyroid origin; TTF-1, origin magnification ×200 (B) and TG, origin magnification ×200 (C).

some areas. Normal bone marrow tissues were extensively replaced by alveolus in lung carcinoma. Cancer cells of thyroid origin were scattered or organized in microfollicular pattern, in the center of which were filled with eosinophilic homogenous material. HCC is one of the most common malignant diseases in China [26, 27], which infiltrated bone marrow with thickened trabecula pattern closely resembling normal liver plates, and bile was observed in some cancer cells. BM-STUOs of stomach carcinomas origin was mainly consisted of signet cells. Squamous cell carcinomas of esophagus and lung presented some similar characteristics: arranging in nests, prominent desmoplastic reaction and keratinization. These histological features are helpful in the diagnosis of primary tumor.

Our study revealed that desmoplastic reaction was significant in most cases, though the density of the reaction was variable. The interaction between the tumor cell and bone marrow microenvironment was another focus in recent studies [28, 29]. According to current dogma, some factors can selectively inhibit cell-cell adhesion by increasing the attachment with extracellular matrix in bone marrow. Several growth signals secreted by stromal cells may act as ligands for the cellular integrins expressed by cancer cells [29]. Tumorous fibrosis, on the one hand, could weaken the invading ability of malignant cells, but, on the other hand, provide some necessary factors for the growth of tumors, and even protect them from the destruction by the host immune response. Whether we can distinguish the tumor desmoplastic reaction from benign fibrosis induced by inflammation, autoimmune disease or idiopathic myelofibrosis etc. and further disclose some pivotal changes in signal pathways is a fascinating problem.

With the improvement of antibody, IHC has become one of the most important tools in clinical pathological diagnosis: the precise identification of metastatic lesions in bone marrow originating in prostate was greatly facilitated by

positive staining for PSA, PSMA and PAP. Both lung carcinoma and thyroid follicular carcinoma were positively stained by TTF-1, but the latter also expressed TG and HBME-1. E-Cadherin expressed in breast invasive ductal carcinoma. Although GCDFP-15 did not specific and only expressed in apocrine sweat glands subgroup, it is another helpful marker for the diagnosis of metastatic breast cancer. The staining pattern of esophageal carcinomas and stomach carcinomas was CK7+/CK20-, which was different from CK7-/CK20+ of colon carcinomas, and both of them could express CEA and CDX-2, which could be used in the differential diagnosis. Positive-staining for Hep Par1, AFP and HBsAg was useful in the diagnosis of metastatic HCC.

However, because the number of monoclonal antibodies with high specificity is limited, more unconventional molecular techniques are expected. Recent studies have paved the way for the employment of microRNA as a promising candidate in identification and classification of tumor tissue origin, because different microRNAs have been tied to the development of specific malignancies [30, 31]. Although the analysis process need large data of gene features and could not correctly identify poorly differentiated cancers at present, fourteen cancer classes can be accurately identified by the means of microRNA microarray platform.

The results of our study showed that, even after a systemic treatment for primary tumor, median survival of patients with BM-STUOs was a mere 11 months. So, it is an ideal status that cancer cells should be detected and even destroyed before the formation of metastatic lesion in bone marrow. Circulating cancer cells (CTCs), which traveled via venous or lymphatic circulation in order to metastasize to bone marrow or other organs, were found in patients with metastatic lesions [32-35]. In a recent analysis of CTCs, tumors with preference of BM metastasis were confirmed to be genetically distinct from BM-negative tumors, and CTCs were presented with the ability to invade bone marrow [36]. It is an intriguing possibility that CTCs must share some molecular characteristics with their normal counterparts. The monoclonal antibody, targeting one specific marker of CTCs for therapeutic purposes, may have an effect on several BM-STUOs.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (No. 81300347), the Natural Science Foundation of Jiangxi Province, China (No. 20132BAB205037, 20151BAB215008, 20151BBG70200), and Foundation of Jiangxi Educational Committee (No. GJJ14192), Foundation of Health and Family Planning Commission of Jiangxi Province (No. 20155592, 20155103, 20161086, 20161093).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Huan Deng, Department of Pathology, The Fourth Affiliated Hospital of Nanchang University, 133 South Guangchang Road, Nanchang 330003, Jiangxi Province, China. E-mail: beandeng@ncu.edu.cn

References

[1] Oeffinger KC and Bhatia S. Second primary cancers in survivors of childhood cancer. Lancet 2009; 374: 1484-1485.

[2] Lu X and Kang Y. Cell fusion as a hidden force in tumor progression. Cancer Res 2009; 69: 8536-8539.

[3] Hoeijmakers JH. DNA damage, aging, and cancer. N Engl J Med 2009; 361: 1475-1485.

[4] Jemal A, Tiwari RC, Murray T, Ghafoor A, Samuels A, Ward E, Feuer EJ and Thun MJ. Cancer statistics, 2004. CA Cancer J Clin 2004; 54: 8-29.

[5] Ringenberg QS, Doll DC, Yarbro JW and Perry MC. Tumors of unknown origin in the bone marrow. Arch Intern Med 1986; 146: 2027-2028.

[6] Sun C, Jin XL and Xiao JC. Oval cells in hepatitis B virus-positive and hepatitis C virus-positive liver cirrhosis: histological and ultrastructural study. Histopathology 2006; 48: 546-555.

[7] Simmons R, Hoda S and Osborne M. Bone marrow micrometastases in breast cancer patients. Am J Surg 2000; 180: 309-312.

[8] Ninomiya J, Oyama T, Horiguchi J, Koibuchi Y, Yoshida T, Iijima K, Yoshida M, Takata D, Iino Y and Morishita Y. Two cases of breast cancer with cartilaginous and osseous metaplasia. Breast Cancer 2005; 12: 52-56.

[9] Jain S, Fisher C, Smith P, Millis RR and Rubens RD. Patterns of metastatic breast cancer in relation to histological type. Eur J Cancer 1993; 29A: 2155-2157.

[10] Yu JJ, Brennan M, Christos P, Osborne MP, Hoda S and Simmons RM. Bone marrow micrometastases and adjuvant treatment of breast cancer. Breast J 2004; 10: 181-185.
[11] Solakoglu O, Maierhofer C, Lahr G, Breit E, Scheunemann P, Heumos I, Pichlmeier U, Schlimok G, Oberneder R, Kollermann MW, Kollermann J, Speicher MR and Pantel K. Heterogeneous proliferative potential of occult metastatic cells in bone marrow of patients with solid epithelial tumors. Proc Natl Acad Sci U S A 2002; 99: 2246-2251.
[12] Bubendorf L, Schopfer A, Wagner U, Sauter G, Moch H, Willi N, Gasser TC and Mihatsch MJ. Metastatic patterns of prostate cancer: an autopsy study of 1,589 patients. Hum Pathol 2000; 31: 578-583.
[13] Hansen HH, Muggia FM and Selawry OS. Bone-marrow examination in 100 consecutive patients with bronchogenic carcinoma. Lancet 1971; 2: 443-445.
[14] Anner RM and Drewinko B. Frequency and significance of bone marrow involvement by metastatic solid tumors. Cancer 1977; 39: 1337-1344.
[15] Usnarska-Zubkiewicz L, Strutynska-Karpinska M, Podolak-Dawidziak M, Nienartowicz M, Grabowski K, Prajs I and Kuliczkowski K. Epithelial bone marrow cells in patients with advanced esophageal squamous cell carcinoma. Neoplasma 2009; 56: 245-251.
[16] Spence GM, Graham AN, Mulholland K, Maxwell P, McCluggage WG, Sloan JM and McGuigan JA. Bone marrow micrometastases and markers of angiogenesis in esophageal cancer. Ann Thorac Surg 2004; 78: 1944-1949; discussion 1950.
[17] Taketo MM. Role of bone marrow-derived cells in colon cancer: lessons from mouse model studies. J Gastroenterol 2009; 44: 93-102.
[18] Pleyer L, Went P, Russ G, Prinz E, Faber V, Rowert HJ, Karlbauer R and Greil R. Massive infiltration of bone marrow in colon carcinoma after treatment with activated protein C. Wien Klin Wochenschr 2007; 119: 254-258.
[19] Sutcliffe R, Maguire D, Murphy P, Portmann B, Rela M, O'Sullivan G, Mufti G and Heaton N. Detection and clinical significance of bone marrow micrometastases in patients undergoing liver transplantation for hepatocellular carcinoma. Transplantation 2005; 80: 88-94.
[20] Kienle P, Weitz J, Klaes R, Koch M, Benner A, Lehnert T, Herfarth C and von Knebel Doeberitz M. Detection of isolated disseminated tumor cells in bone marrow and blood samples of patients with hepatocellular carcinoma. Arch Surg 2000; 135: 213-218.
[21] Clark JR, Lai P, Hall F, Borglund A, Eski S and Freeman JL. Variables predicting distant metastases in thyroid cancer. Laryngoscope 2005; 115: 661-667.
[22] Yilmazlar T, Kirdak T, Adim S, Ozturk E and Yerci O. A case of hemangiosarcoma in thyroid with severe anemia due to bone marrow metastasis. Endocr J 2005; 52: 57-59.
[23] Mirallie E, Vuillez JP, Bardet S, Frampas E, Dupas B, Ferrer L, Faivre-Chauvet A, Murat A, Charbonnel B, Barbet J, Goldenberg DM, Chatal JF and Kraeber-Bodere F. High frequency of bone/bone marrow involvement in advanced medullary thyroid cancer. J Clin Endocrinol Metab 2005; 90: 779-788.
[24] Janni W, Rack B, Lindemann K and Harbeck N. Detection of micrometastatic disease in bone marrow: is it ready for prime time? Oncologist 2005; 10: 480-492.
[25] Muller V and Pantel K. Bone marrow micrometastases and circulating tumor cells: current aspects and future perspectives. Breast Cancer Res 2004; 6: 258-261.
[26] Plymoth A, Viviani S and Hainaut P. Control of hepatocellular carcinoma through hepatitis B vaccination in areas of high endemicity: perspectives for global liver cancer prevention. Cancer Lett 2009; 286: 15-21.
[27] Shi YH and Shi CH. Molecular characteristics and stages of chronic hepatitis B virus infection. World J Gastroenterol 2009; 15: 3099-3105.
[28] Yoneda T and Hiraga T. Crosstalk between cancer cells and bone microenvironment in bone metastasis. Biochem Biophys Res Commun 2005; 328: 679-687.
[29] Roodman GD. Mechanisms of bone metastasis. N Engl J Med 2004; 350: 1655-1664.
[30] Rosenfeld N, Aharonov R, Meiri E, Rosenwald S, Spector Y, Zepeniuk M, Benjamin H, Shabes N, Tabak S, Levy A, Lebanony D, Goren Y, Silberschein E, Targan N, Ben-Ari A, Gilad S, Sion-Vardy N, Tobar A, Feinmesser M, Kharenko O, Nativ O, Nass D, Perelman M, Yosepovich A, Shalmon B, Polak-Charcon S, Fridman E, Avniel A, Bentwich I, Bentwich Z, Cohen D, Chajut A and Barshack I. MicroRNAs accurately identify cancer tissue origin. Nat Biotechnol 2008; 26: 462-469.
[31] Shedden KA, Taylor JM, Giordano TJ, Kuick R, Misek DE, Rennert G, Schwartz DR, Gruber SB, Logsdon C, Simeone D, Kardia SL, Greenson JK, Cho KR, Beer DG, Fearon ER and Hanash S. Accurate molecular classification of human cancers based on gene expression using a simple classifier with a pathological tree-based framework. Am J Pathol 2003; 163: 1985-1995.
[32] Sierra A. Metastases and their microenvironments: linking pathogenesis and therapy. Drug Resist Updat 2005; 8: 247-257.

[33] MacDonald IC, Groom AC and Chambers AF. Cancer spread and micrometastasis development: quantitative approaches for in vivo models. Bioessays 2002; 24: 885-893.
[34] Rubio N, Espana L, Fernandez Y, Blanco J and Sierra A. Metastatic behavior of human breast carcinomas overexpressing the Bcl-x(L) gene: a role in dormancy and organospecificity. Lab Invest 2001; 81: 725-734.
[35] Chambers AF, Naumov GN, Varghese HJ, Nadkarni KV, MacDonald IC and Groom AC. Critical steps in hematogenous metastasis: an overview. Surg Oncol Clin N Am 2001; 10: 243-255, vii.
[36] Zetter BR and Banyard J. Cancer. The silence of the genes. Nature 2002; 419: 572-573.