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Original article

Immunohistochemical evaluation of bone metastases

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Introduction. Metastases are the most common type of malignancy involving the bone, while bone is the third most frequent site for metastases, after the lung and liver. In some patients, medical history, physical and laboratory examination are not conclusive to identify the primary tumor site. In such cases a bone biopsy and immunohistochemical analysis may contribute to the diagnosis, determination of appropriate treatment and evaluation of prognosis. In this study, we tried to evaluate the imunochistochemical expression in bone metastases.

Material and methods. We reviewed 125 patients, with a mean age of 63 years, treated for bone metastases in our institution. All patients received palliative orthopaedic surgery for bone metastatic carcinoma. Fifty-eight patients had already an established diagnosis of the primary tumor, while 67 patients presented metastases with an unknown primary tumor origin. Immunohistochemical analysis was performed to intra-operative bone biopsy specimens. The expression of cytokeratine 7, cytokeratin 20 and the expression of a panel of other organ-specific markers were recorded. In patients with a known primary tumor, we examined the relationship between the origin of metastases, as suggested by the cytokeratin phenotype, compared with the one indicated by the initial histological diagnosis. We also recorded the efficacy of organ-specific markers to identify the primary tumor origin in epithelial bone metastases and we evaluated the prognosis between patients with a immunohistologically determined primary tumor origin, with those with an undetermined one.

Results. Associations of cytokeratine 7 and cytokeratine 20 expression confirmed diagnosis in 51 out of the 58 patients (88%) with a known primary tumor (Cohen's K test 0.79 SE 0.80, P < 0.0005). Immunohistochemical analysis also contributed to establish the diagnosis of patients with an unknown primary tumor, yielding diagnosis in 35 out of the 67 cases (52%). Patients with an immunochistologically undetermined primary tumor site presented a statistically significant poorer prognosis.

Conclusions. Cytokeratine 7 and cytokeratine20 are useful immunochistochemical markers in determining a preliminary evaluation of bone metastases. Organ-specific immunohistochemical markers have a reliable role in either suggesting or confirming the possible origin of metastases. An indeterminate immunohistochemical phenotype seems to relate to a less differentiated lesion, with a worse prognosis.

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Key words: bone metastases, immunohistochemistry, cytokeratine 7, cytokeratine 20, organ specific markers, cancer of unknown primary origin

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Introduction

Metastasis is the most common type of malignancy involving the bone, whereas bone represents the third most frequent site for metastases, after the lung and liver [1–3]. Bony lesions may be the first manifestation of malignancy in 25–30% of cases, and sometimes the only manifestation; as in metastatic disease of unknown origin [1]. The number of cancer survivors has been rising in recent years. The 5-year survival rate in breast and colorectal cancer is reported to be more than 60% and 85%, respectively. Accordingly, the 5-year survival rate for patients with prostate cancer is reported to be more than 95%. In this setting, the increase in the overall survival in cancer patients may result in an increase in the overall incidence of bone metastases [4, 5].

In general, previous medical history, physical examination, laboratory tests, and imaging modalities are usually sufficient to establish a diagnosis of bone metastases. Differential diagnosis includes primary malignant bone tumors (i.e. sarcoma or chondrosarcoma), hematologic diseases (i.e. multiple myeloma or lymphoma) and osteomyelitis. However, in cases of occult primary malignancies, diagnosis may be difficult and bone biopsy and histology may be necessary to determine the appropriate treatment and prognosis [1]. Determination of the type and origin of metastatic disease constitutes a challenging area in pathology. A microscopical evaluation may reveal specific morphological features suggestive of tumor lineage and origin. However, in the absence of distinctive characteristics, immunohistochemichal (IHC) evaluation may help in diagnosis. A panel of tissue- or organ-specific markers is typically used. Thus, many authors underline the important role that cytokeratines and other specific markers play in the discrimination of primary tumor lineage and determination of metastatic disease [6–13].

In this context, aiming to provide further insights concerning IHC marker expression in metastatic carcinomas, we reviewed bone-tissue biopsies in a series of patients with bone metastases. Our purpose was to evaluate the effectiveness of these specific markers in identifying (or verifying) the primary tumor site and determine if they can provide certain information concerning prognosis.

Materials and methods

We initially reviewed the medical files of 194 patients who were admitted and treated for bone metastases to our institution. All patients received palliative orthopaedic surgery. Pathology reports from intra-operative specimens of bone metastases were reviewed. 35 patients were excluded, because their primary tumor was other than carcinoma. Likewise, 34 patients were excluded because of incomplete clinical or pathological data in their files. A total of 125 patients (75 men and 50 women) were finally evaluated. The mean age of primary tumor diagnosis was 58.6 years (range, 33–86 years), while bone metastases

Table I. Anatomical distribution of bone metastases (in the case of multiple metastases, all sites were included; bone metastases in more than three sites were defined as diffuse)

Skeletal site	Patients (n)
Humerus	32
Radius	5
Femur	40
Tibia	10
Spine	22
Pelvis	17
Tarsus	0
Clavicle	4
Scapula	7
Diffuse	5

were diagnosed at a mean age of 63 years (range, 39–87 years). The time lapse from primary diagnosis of malignancy to the development of bone metastases was 40 months (range, 9–304 months). 51 patients (40.5%) presented bone metastases at a point after the primary tumor diagnosis, while 74 patients (59.5%) had already bone metastases at the time of initial diagnosis. Histological diagnosis of the primary tumor was already established at the time of the bone biopsy in 58 cases. In 84 patients (67%), pain was the only clinical sign of metastases development. The mean time between onset of symptoms and metastases detection was 6 months (range, 1–50 months). 30 patients (24%) presented a pathological fracture. The mean survival after bone metastasis detection was 14 months (range, 1–50 months).

An imaging work-up (plain radiographs, CT scans, MRI scans, bone scans) was reviewed to locate the sites of metastases, the nature of the lesions and their relationship with the adjacent tissues. Table I and Table II show the anatomical distribution of bone metastases and the primary tumor origin in our patients. Pathology reports of biopsies from bone metastases were reviewed in terms of IHC analysis. Tumor tissues were collected during surgical treatment of the bone lesion, and were then fixed for 2–5 days in neutral buffered formalin and then processed for decalcification protocol and for paraffin embedding. Fixed specimens were decalcified in 20% EDTA in 10N NaOH at pH 7.4 from several days up to several weeks with intermittent shaking to make a sure the solution was flowing around the bone. The decalcification time depended on the degree of mineralisation and the size of the specimen. Representative 5 μm thick sections were mounted on a glass slide covered with 2% silane solution in acetone. After dewaxing in xylene and rehydration in ethanol, for standard histology, slides were stained with Hematoxylin & Eosin staining. For immunoistochemistry, slides were processed for antigen retrieval by heating in

Table II. A breakdown of the primary tumors in our patients with bone metastases

Primary tumor	Patients with bone metastases (n)	Percentages
Unknown	67	53.6%
Lung	5	4%
Kidney	16	12.8%
Breast	19	15.2%
Uterus	3	2.4%
Esophagus	2	1.6%
Liver	2	1.6%
Colon Rectum	1	0.8%
Stomach	2	1.6%
Cervix	1	0.8%
Prostate	2	1.6%
Biliary tract	1	0.8%
Small intestine	1	0.8%
Bladder	3	2.4%
Total	125	100%

a microwave oven in 0.02 M citrate buffer, pH 6.0. After cooling, tissue sections were incubated in 3% perhydrol solution to block the endogenous peroxidase reaction. Non-specific binding was blocked by incubation in 5% bovine serum albumin. Incubation with specific primary antibodies was then followed by incubation with a biotinylated secondary antibody. Sections were then covered with DAB and counterstained with Mayer's hematoxylin. Negative controls were also performed by omitting the primary antibody. The expression of cytokeratine 7 (CK7), the expression of cytokeratin 20 (CK20) and the expression of a panel of other organ-specific markers, according to the expected tissue//organ of origin, were recorded. CEA and CDX2 were examined for gastrointestinal adenocarcinoma, APS and FAP

for prostate adenocarcinoma, ER, PGR, GCDFP for breast carcinoma, TTF1 for pulmonary adenocarcinoma, WT1 for renal adenocarcinoma, HEP for hepatic carcinoma, and TIR for thyroid adenocarcinoma.

In the patients with an already known primary tumor, we examined the relationship between the origin of metastases, as suggested by cytokeratin phenotype (CK7/CK20), compared with the one indicated by the initial histological diagnosis. Subsequently, we examined all the patients of the study evaluating the relationship between the origin of metastases, as suggested by the organ-specific IHC markers, and the one indicated by histological diagnosis. The expected and actual results obtained by IHC markers have been assessed by Cohen's K test, AUC ROC, sensitivity and specificity.

Statistical analysis was performed with SPSS v. 19. Comparison between the groups of patients was conducted with Pearson's Chi Square Test, calculated with the Monte Carlo method for small samples. Cohen's K test and ROC analysis were conducted to compare the estimated and effective values between the groups. The Kaplan-Meier survival analysis was performed, while the Breslow test was conducted to assess the different features of each group.

Results

The IHC results in bone metastasis specimens from hepatic carcinoma (2 patients; one identified by the phenotype CK7+/CK20+ and the other by CK7+/CK20–) and small intestine carcinoma (1 patient; identified by the phenotype CK7–/CK20–) were not significant and were excluded. Hence, cytokeratin expression was evaluated in 55 cases out of the 58 cases with a known primary tumor in the Pearson's Chi Square test. Results of the cytokeratin phenotype in metastatic bone disease are shown in Table III; agreement between the cytokeratine phenotype and the primary histology of already diagnosed tumors is shown in Table IV. Results of Cohen's K test, AUC ROC, the sensitiv-

Table III. Cytokeratin phenotype expression in bone metastases specimens according to primary tumor sites. Patients with an unknown primary tumor site were excluded

Metastases origin	Patients (n)	No. of cases responsive to CKs test	CK7	CK20	Significance
Biliary tract	1	1 (100%)	+	+	P < 0.005
Bladder	3	3 (100%)	+	+	
Breast	19	18 (95%)	+	–	
Uterus	3	3 (100%)	+	–	P < 0.005
Esophagus	2	2 (100%)	+	–	
Lung	5	4 (80%)	+	–	
Stomach	2	1 (50%)	–	+	
Cervix	1	1 (100%)	–	+	P = 0.03
Colon rectum	1	1 (100%)	–	+	
Prostate	2	2 (100%)	–	–	P < 0.005
Kidney	16	14 (87.5%)	–	–	

Table IV. Relationship between primary histological results with cytokeratin expression in bone metastases. Patients with an unknown primary tumor site were excluded

Agreement between primary histology & cytokeratin Phenotype in bone metastases	CK7+/CK20+	CK7+/CK20–	CK7–/CK20+	CK7–/CK20–
Significant results	Biliary tract*	Lung*	Colon — rectum°	Kidney*
	Bladder*	Breast*	Stomach°	Prostate*
		Uterus*	Cervix°	
		Esophagus*		
Not significant results	Prostate	Kidney		Breast
		Prostate		Uterus
		Bladder		

°Pearson's Chi Square test presented statistical significance (P < 0.05); *Pearson's Chi Square test presented high statistical significance (P < 0.005)

Table V. Results of Cohen's K test, AUC ROC, the sensitivity and specificity between expected and actual values of IHC (primary tumor and metastasis). Patients with an unknown primary tumor site were excluded

	CK 7+/CK 20+	CK 7+/CK 20–	CK 7–/CK 20+	CK 7–/CK 20–
Sensibility	100%	93%	100%	80%
Specificity	98%	88%	100%	93%
Cohen's K	0.85 — SE 0.15*	0.80 — SE 0.08*	1 — SE 0.0°	0.75 — SE 0.96*
AUC ROC	0.99 ± 0.01*	0.90 ± 0.05*	1 ± 0.0#	0.87 ± 0.06*

*P < 0.001; °P < 0.05; #P > 0.05

ity and specificity between expected and actual values of immunochistochemistry (primary tumor and metastases) are shown in Table V. Accordingly, patients with unknown primary tumor origin were excluded. The closer Cohen's K value was to 1, the greater the probability that metastases came from the indicated site of the cytokeratin phenotype.

ICH verification was observed in 50 out of 58 patients (86.2%) with known primary tumor origin. Cohen's K agreement between the primary tumor histology and the IHC results of bone metastases was 0.79 — SE 0.80, P < 0.0005.

In 32 out of 67 patients (48%) though, with unknown primary tumor origin, IHC identification was not possible; in

Table VI. Primary tumor origin and organ-specific IHC markers for epithelial metastases

Primary tumor	Number of metastases	Diagnosis of tumor origin after organ-specific IHC markers test on bone metastases							
		Unknown	Lung	Kidney	Breast	Liver	Rectum	Stomach	Prostate
Unknown	67	32	12	6	7	–	4	2	4
Lung	5	3	2	–	–	–	–	–	–
Kidney	16	2	–	14	–	–	–	–	–
Breast	19	2	–	–	17	–	–	–	–
Uterus	3	3	–	–	–	–	–	–	–
Esophagus	2	1	1	–	–	–	–	–	–
Liver	2	–	–	–	–	2	–	–	–
Rectum	1	–	–	–	–	–	1	–	–
Stomach	2	–	–	–	1	–	–	1	–
Cervix	1	–	–	–	–	–	1	–	–
Prostate	2	–	–	–	–	–	–	–	2
Biliary Tract	1	1	–	–	–	–	–	–	–
Small Intestine	1	–	–	1	–	–	–	–	–
Bladder	3	3	–	–	–	–	–	–	–
TOTAL	125	47	15	21	25	2	6	3	6

Table VII. The table shows the survival rate of patients based on the IHC results in bone metastases (patients with lung, renal and breast primary carcinoma)

Metastases site of origin	Results with IHC markers	Survival	
		No. pts with survival < 12 months	No. pts with survival > 12 months
Lung	Undetermined	3	–
	Determined	1	1
Kidney	Undetermined	1	–
	Determined	4	11
Breast	Undetermined	2	1
	Determined	5	11

Figure 1. Patients with undetermined results in the ICH evaluation of bone metastases had a poorer 1-year survival rate than those with the determined phenotype (P = 0.007 at Breslow test)

the remaining 52%, IHC identification was achieved (Tab. VI). A false negative ICH result was provided in 3 out of 5 cases (60%) with lung carcinoma. Likewise, a false negative result was provided in 2 out of 16 patients (12.5%) with renal carcinoma, and in 2 out of 19 patients with breast carcinoma (10.5%). The majority of patients in this series presented lung, renal or breast carcinoma. Table VII shows the relationship between the 1-year survival of these patients and their corresponding IHC expression in bone metastases. It should be noticed that 3 out of 4 patients (75%) with bone metastases from lung carcinoma that died within a year, had an undetermined IHC phenotype. On the other hand, only 4 out of 15 patients (26.6%) with IHC determined metastatic renal carcinoma and 5 out of 16 patients (31.2%) with determined metastatic breast carcinoma died within a year. Overall, patients with undetermined IHC results in bone metastases had a worse survival rate than those with determined results (P = 0.007, Breslow test; Fig. 1). Gender, an unknown primary tumor location, and the presentation of a pathological fracture, were not found to be statistically significant prognostic factors in our series (P > 0.05, Breslow test).

Discussion

Cytokeratins consist of a family of over 20 polypeptides. They are intermediate filament proteins in epithelial cells that may be used to identify epithelial differentiation, as their expression pattern is usually preserved in neoplastic cells [11, 14]. Monoclonal antibodies against cytokeratin polypeptides have been established to evaluate their tissue-specific expression. Cytokeratins have been reported to be effective in distinguishing between various subsets of primary and metastatic carcinomas in different anatomical sites. More specifically, many authors have studied the co-ordinate expression of CK7 and CK20 and reported the high sensibility and specificity of their association [6–13]. In our study we found that when these markers are both positive, both negative or when CK7 is positive and CK20 is negative there is relative success in finding possible origins in finding
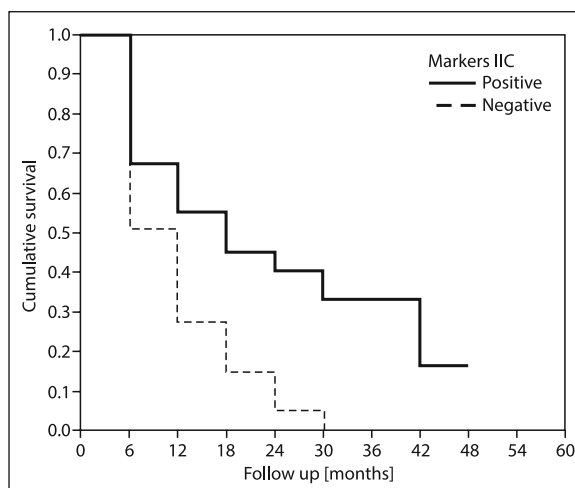
possible origins for bone metastasis from carcinoma. Additionally, we observed that the combination of a negative CK7 and a positive CK20 was not considered as accurate in diagnosis (P > 0.05) as in the other cases.

A combination of CK7 and CK20 has been previously reported to provide effective discrimination between carcinomas and is often used as part of a panel with other tissue- or organ-specific IHC markers to establish the primary site of malignancy [12]. An algorithmic and probabilistic approach and the use of a detailed database have been recommended in previous reports for effective assessment [8]. In a recent study [12], it has been suggested that CK7+/CK20+ may represent the profile of urothelial carcinoma, pancreatic ductul adenocarcinoma, ovarian mucinous carcinoma, bladder adenocarcinoma, gastric adenocarcinoma or cholangiocarcinoma. Accordingly, the CK7+/CK20– phenotype may indicate lung adenocarcinoma, lung small cell carcinoma, breast carcinoma, ovarian serous carcinoma, endometrial adenocarcinoma, cholangiocarcinoma, thyroid carcinoma, mesothelioma, pancreatic carcinoma or gastric carcinoma. In the same setting, CK7–/CK20+ expression may refer to colorectal adenocarcinoma or Merckel cell carcinoma, while CK7–/CK20– to prostate adenocarcinoma, renal cell carcinoma, hepatocellular carcinoma, squamous cell carcinoma, adrenal cortical carcinoma or gastric adenocarcinoma [12]. However, it should be mentioned that difficulties in discrimination often occur, as the cytokeratin phenotype may be altered in metastatic disease as compared to the primary tumor origin [8].

IHC determination of the site of origin of carcinomas is often achievable. When a metastasis is determined to be a carcinoma on the basis of screening immunostains, a panel of tissue- or organ-specific markers can be used in an attempt to suggest (or confirm) the tumor origin. How-

ever, diverse tumor-or organ-specific ICH markers may also react with variable tumor types. In this setting, in case of metastatic tumors of unknown origin, the use of a panel of IHC markers is strongly suggested [12]. In our study we used such a IHC panel in 67 patients who had bone metastases from an unknown primary origin. However, in 32 of these patients (47%) the tumor origin remained undetermined. An explanation to this may be the fact that metastatic tumors can change geno- or phenotype. Hence, different immunoprofiles of primary carcinomas and their metastasis may exist [15]. On the other hand, carcinomas of the unknown primary site represent, by themselves, a separate clinical entity that accounts for about 5% of all cancers [16].

The subset of patients in our study, where IHC analysis did not determinate the primary tumor site, were found to present a poorer prognosis. Diagnosis of a metastatic carcinoma of an unknown primary site has long been considered synonymous with poor prognosis, with a median overall survival time range from 6 to 13 months. Empiric chemotherapy is generally administered in these patients, while the benefit of different treatments studied compared with best supportive care is still unclear [17]. The expression of the determinate IHC phenotype in high grade tumors might correspond to a better differentiated appearance, with a better prognosis. Considering these facts, even if further studies with wider populations are required, patients with an undifferentiated phenotype may have a poorer prognosis.

Conclusions

CK7 and CK20 provide effective discrimination between carcinomas in bone metastasis specimens. They are often used as part of a panel with other tissue- or organ-specific IHC markers to establish the primary tumor site. A combination of these markers when examined in tissue of bone metastases may determine the origin of the primary tumor; however in some patients, primary malignancy will not be identified. The latter patients are expected to present a poorer prognosis.

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