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Immunocytochemical Detection of Epithelial Cells in Bone Marrow of Carcinoma of the Upper GI tract: Incidence, Clinical Significance, and Prognostic Relevance

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Key Words
Epithelial cells, bone marrow · Upper GI tract, carcinoma · Immunocytochemistry · Prognosis (carcinoma, upper GI tract)

Summary
In this review, the influence of epithelial cells detected in bone marrow of patients with carcinoma of the upper GI tract on prognosis is analyzed. Methodological variables affecting bone marrow sampling, immunocytochemical approaches, and phenotyping of epithelial cells were evaluated as well as the correlation to clinical factors. The detection of epithelial cells in bone marrow varies between 25 and 82% in gastric cancer patients and is about 40% in esophageal cancer patients. Univariate analysis revealed that the incidence of epithelial cells in bone marrow is correlated in gastric cancer patients to the Laurén classification, to the lymph node involvement, and to an increased recurrence rate. First results of phenotyping epithelial cells showed that uPA receptor expression is correlated with clinical prognosis in gastric cancer patients. The finding of cytokeratin (CK)-positive cells in bone marrow of esophageal cancer patients is predictive of a reduced relapse-free and overall survival indicating a hematogenous dissemination of viable malignant cells leading to an increased risk of metastatic relapse. Preliminary investigations showed that epithelial cells are tumorigenic and resistant to neoadjuvant therapy in GI tract cancer. In conclusion, immuncytologically and phenotyping of CK-positive cells might be helpful for tumor staging and to monitor the response of neoadjuvant therapy, but the results should be confirmed in multivariate analysis.

Schlüsselwörter
Epitheliale Zellen, Knochenmark · Oberer GI-Trakt, Karzinom · Immunzytochemie · Prognose (Karzinom, oberer GI-Trakt)

Zusammenfassung
In der vorliegenden Übersichtsarbeit wird der Stellenwert epithelialer Zellen, die im Knochenmark von Patienten mit Karzinomen des oberen Gastrointestinaltrakts nachgewiesen wurden, analysiert. Neben Faktoren, die bei der Gewinnung des Knochenmarkaspirats, zum immunzytochemischen Nachweis oder der Phänotypisierung der epithelialen Zellen von Bedeutung sind, wurde auch die Korrelation zu klinischen Prognosefaktoren überprüft. Die Nachweisrate von epithelialen Zellen im Knochenmark liegt zwischen 25 und 82% beim Magenkarzinom und um 40% beim Oesophaguskarzinom. In einer univariaten Analyse zeigte sich, daß der Nachweis epithelialer Zellen im Knochenmark beim Magenkarzinompatienten mit der Laurén-Klassifikation, dem Nachweis von Lymphknotenmetastasen und einer erhöhten Tumorrezidivrate korreliert. Erste Ergebnisse bezüglich der Phänotypisierung epithelialer Zellen zeigen, daß die Expression des uPA-Rezeptors beim Magenkarzinompatienten mit dem weiteren klinischen Verlauf korreliert. Beim Oesophaguskarzinompatienten ist der Nachweis epithelialer Zellen im Knochenmark verbunden mit einer verkürzten Gesamt- und rezidivfreien Überlebenszeit des Patienten. Außerdem scheint eine Resistenz der im Knochenmark nachgewiesenen disseminierten, epithelialen Zellen gegenüber etablierten neoadjuvanten Chemotherapieverfahren vorzuliegen. Ob der Nachweis und die Phänotypisierung Cytokeratin-positiver Zellen aus dem Knochenmark bei Patienten mit einem Tumor des oberen Gastrointestinaltrakts zum Tumorstaging und Monitoring bei neoadjuvanter Therapie hilfreich ist, muß noch anhand multivariater Analysen bestätigt werden.

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Introduction

Even after apparently curative surgery in patients with carcinoma of the upper gastrointestinal tract, a large number of patients will experience disease recurrence [1–4]. It is assumed that these patients already had occult micrometastases at time of primary surgery. In various types of carcinomas the incidence of epithelial cells in bone marrow may be an indicator of the disseminatory potential of individual tumors [5–13]. Therefore, the standardization committee of the International Union Against Cancer (UICC) has suggested that a new subcategory, called pM1(i) should be incorporated into the existing tumor classification scheme [14]. In the present review, an evaluation of the frequency, clinical significance and prognostic relevance concerning epithelial cells in bone marrow of patients with carcinoma of the upper GI tract was performed.

Methodological Aspects: Aspiration Sampling, Immunocytology, and Phenotyping

In all studies bone marrow is used as the indicator organ because it is easily accessible and is normally devoided of epithelial cells. Aspirates are obtained from the upper iliac crest and/or sternum through an aspiration needle. The volumes of all aspirates range from 2 to 5 ml, yielding between 8×10^5 and 2×10^6 mononuclear cells ml. To obtain a representative sample, analysis of 2 aspirates from both sites of the iliac crest seems to be sufficient to detect about 90% of patients with epithelial cells in bone marrow [15]. In contrast, recent results showed that detection rates based on iliac crest marrow are underestimated. O'Sullivan et al. [16] found epithelial cells in bone marrow of the rib in 88% of 50 patients with carcinoma of the esophagogastric junction.

After centrifugation through a Ficoll-Hypaque density gradient, interface cells are cytocentrifuged onto glass slides. The cytocentrifuge preparations are fixed with acetone and then preincubated with antibody-free human serum to block nonspecific antibody binding. Epithelial cells in bone marrow were identified immunocytologically using different anti-cytokeratin monoclonal antibodies (mAbs) directed against cytokeratin (CK) polypeptides [17, 18]. The antibody reaction is employed with the alkaline phosphatase-antialkaline phosphatase (APAAP) technique for visualizing antibody binding [20]. A bone marrow preparation is scored positive when a strong red color reaction in 1 or more stained cells is observed (fig. 1). In several studies non-carcinoma control patients served as control specimens to test for the specificity of the immunostaining. Furthermore, model studies on immunocytochemical assays have shown that even single epithelial cells can be detected in 95% of samples at a concentration of 2×10^6 mononuclear cells [21].

At present additional information on the malignant potential of epithelial cells can be obtained by double marker analysis. The double marker analysis is helpful to characterize the epithelial CK-positive cells detected in bone marrow by phenotyping for cellular proteins. Hereby, the development of metastasis is focused on enhanced cell motility, changes in the expression of adhesion molecules and proteases, or enhanced cell motility of epithelial cells in bone marrow.

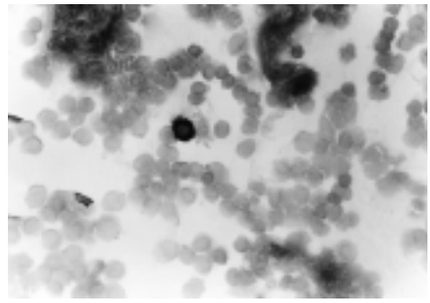

Fig. 1. Cytokeratin-positive epithelial cell in a bone marrow cytospin preparation from a patient with squamous cell carcinoma of the esophagus.

Table 1. Immunocytochemical phenotyping of epithelial cells detected in bone marrow from patients with gastric carcinoma [22–25]

Marker	Marker-positive / CK-positive patients	
	n	%
Ki-67	0/21	
p120	4/13	30.8
ErbB2	6/22	27.3
HLA class I	3/11	27.3
uPA-R	20/44	45.0
E-cadherin	1/9	11.1
p53	4/15	26.6
Plakoglobin	4/13	30.8

The results of immunocytochemical phenotyping marker expression in CK-positive patients with carcinoma of the upper GI tract are presented in table 1 [22–25].

Frequency and Incidence

The prevalence of epithelial cells in bone marrow from patients with carcinoma of the upper GI tract ranges from 25% to over 80%, and the number of cells analyzed from 1×10^5 to 1×10^6 cells per patient (table 2) [6, 23–29]. When a number of detected cells was given, most of the positive marrow samples contained fewer than 10 CK-positive cells. The majority of specimens in our study showed also isolated epithelial cells, whereas clusters of positive cells were observed in the samples of only 5% of all patients [10, 11, 29, 30].

Prognostic Factors

A comparison between the detection of epithelial cells in bone marrow of patients with carcinoma of the upper GI tract and established predictors of clinical outcome are reviewed in the following section. In 1991 Schlimok et al. [6] showed that the incidence of epithelial cells in gastric cancer patients was positively correlated to the histological classification of Laurén, the locoregional lymph node involvement, and an increased relapse rate. Another study in 180 gastric cancer patients from Jauch et al. [7] demonstrated that the prevalence of positive bone marrow findings increased with the wall penetration of

Table 2. Patient collectives, frequency and incidence of epithelial cells in bone marrow from patients with carcinoma of the upper GI tract

Authors	Reference	Primary tumor type	Patients, n	Incidence, %	Cells analyzed, n
Schlimok et al.	[6]	gastric cancer	97	35.0	1.5×10^5
Jauch et al.	[7]	gastric cancer	180	53.0	1.0×10^6
Broll et al.	[26]	gastric cancer	20	82.0	5.0×10^5
Funke et al.	[24]	gastric cancer	102	44.1	1.0×10^6
Maehara et al.	[25]	gastric cancer	46	32.6	1.0×10^5
Juhl et al.	[27]	gastric cancer	48	52.0	2.5×10^5
Thorban et al.		gastric cancer	40	25.0	5.0×10^5
O'Sullivan et al.	[16]	AEG[1]	30	93.3	0.5×10^6
Thorban et al.		AEG[1]	51	39.2	8.0×10^5
O'Sullivan et al.	[16]	SCC[2]	20	80.0	0.5×10^6
Izbicki et al.	[28]	SCC[2]	68	36.8	–
Thorban et al.	[29]	SCC[2]	117	41.1	8.0×10^5

[1] AEG = Adenocarcinoma of the esophago-gastric junction [35];
[2] SCC = Squamous cell carcinoma of the esophagus.

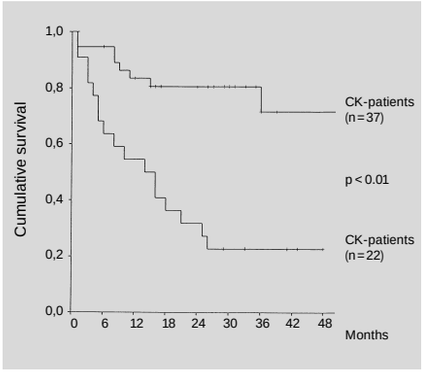

Fig. 2. Overall survival for 59 patients with completely (R0) resected esophageal cancer according to the presence (+) or absence (–) of CK-positive cells in their bone marrow [29].

the primary tumor (pT category) and was also correlated in the follow-up with a higher recurrence rate and a shortened disease-free and overall survival. These findings were supported by Maehara et al. [25], who described that the presence of epithelial cells correlated with tumor differentiation and the depth of tumor invasion. In contrast to the study of Schlimok et al. [6], who found 12.5% of the CK-positive patients with tumor-free lymph nodes (pN0 stage), they reported a rate of 34.8% CK-positive patients without lymph node metastasis.

The impact of bone marrow status on overall and relapse-free survival was evaluated with univariate analysis in most studies. In our study univariate analysis revealed that the presence of CK-positive cells in bone marrow of esophageal cancer patients was predictive of reduced disease-free and overall survival (fig. 2). In addition, recent studies to culture epithelial cells from rib marrow showed a tumorigenic effect in nude mice [16], and the first study in gastric cancer patients published by Heiss et al. [23] demostrated that urokinase plasminogen activator (uPA)-receptor expression on epithelial cells was significantly correlated with clinical prognosis.

To sum it up it can be said that the detection of even single epithelial cells in bone marrow of patients with carcinoma of the upper GI tract indicates the disseminative capability of an individual tumor, but may not predict the site of metastatic growth. For follow-up analyses, only patients who received a complete resection of the primary tumor (R0) should be considered. Furthermore, for immunocytochemical analysis only patients with the same histological primary tumor type should be compared because of the different metastatic behavior of e.g. adenocarcinoma and squamous cell carcinoma. Also the functional clearance of the bone marrow has not been clarified until now. Does there exist a wash out time for epithelial cells and how long will this situation continue?

Therapeutic Strategies

In the search for more specific therapies to eliminate metastatic cells, various modalities of chemotherapy and immunotherapy have been tried. The efficacy of mAb 17–1A in colorectal cancer patients has been published [31], but so far there is no precise prediction of the clinical or histopathological response to neoadjuvant or therapy. A preliminary investigation by O'Sullivan et al. [16] described the resistance of epithelial cells to neoadjuvant therapy, similar results were presented by Solomayer et al. [32] in breast cancer patients and in our experience, 10 of 15 CK-positive esophageal cancer patients and all gastric cancer patients before and after neoadjuvant therapy each displayed epithelial cells in bon marrow (unpublished observations). The majority of epithelial cells in bone marrow appear to be non-cycling and so tend to be resistant to chemotherapeutic agents. Therefore, the detection of CK-positive cells might be helpful to monitor the response and efficacy of neoadjuvant therapy in patients with upper GI tract cancer.

Concluding Comments

The detection of epithelial cells in bone marrow of patients with carcinoma of the upper GI tract as an indicator of generalized tumor dissemination and tumor relapse is a very important aim in clinical oncology. Regarding the prognostic relevance of the selected studies mentioned above, a positive association between the detection of epithelial cells in bone marrow and relapse-free or overall survival remains to be

substantiated by a multivariate analysis. Evaluation of histopathologic factors and the presence of epithelial cells revealed a close association to Laurén's classification and pN category in gastric cancer, but no correlation to conventional prognostic factors in esophageal cancer patients. The lack of correlation between primary tumor stage and the incidence of epithelial cells in bone marrow of esophageal cancer patients can be explained by assuming that cells have the capacity to leave the primary tumor early in its development.

The result of the immunocytochemical detection of epithelial cells in bone marrow largely depends on the method applied. To improve the diagnostic precision of the immunocytochemical assay, morphological evaluation and adequate controls are necessary to prove the specificity of epithelial cells in bone marrow [33].

Reasons for false-negative results are aberrant antigen expression, the number of antibodies used, variations in sample size, contamination with peripheral of sinusoidal blood, and other immunoreactive cells. The heterogeneity of epithelial cells and their resemblance to hematopoetic cells can be problematic. The combination of anti-CD45 mAb, anti κ/λ, and anti-glycophorin A stained nearly all the isolated mononuclear cells and would detect most of the false-positive hematopoetic cells [33]. A standard protocol presented by Pantel et al. [34] may diminish methodological differences to compare prospective studies. At present immunocytology applying mAbs to cytokeratin is a highly sensitive method to predict metastatic potential in upper GI tract cancer and serves as gold standard for assessing new techniques.

In the future more specific information of the biological properties of epithelial cells detected in bone marrow is needed so as by the fluorescence in situ hybridization technique or improved PCR-based assays. Furthermore, more research has to focus on the question whether epithelial cells surviving in bone marrow after neoadjuvant therapy or antibody infusions have the same metastatic potential as cells present before therapy.

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