THE INFLUENCE OF IMMUNOHISTOCHEMICAL FACTORS OF BONE MARROW MICROMETASTASES ON LUNG CANCER PATIENT SURVIVAL RATE AFTER SURGICAL TREATMENT

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The detection of micrometastases in patients with operable non-small cell lung carcinoma (NSCLC) could have a considerable influence on the choice of a proper treatment.

The aim of the study was to evaluate the usefulness of microscopic examination and immunohistochemistry for the detection of micrometastases or single malignant cells in the bone marrow of patients undergoing surgery for NSCLC, as their late survival and recurrence-free time is dependent on immunohistochemical markers of cancer metastases in the bone marrow.

Material and methods. Thirty-five patients were included in the study. Their age range was from 47 to 78 years old. Bone marrow was obtained from a rib during surgery for lung cancer. Both a resected tumour and bone marrow sample were tested for the presence of cytokeratins AE1/AE3, CAM 5.2, CK-7, and CK-18 and other indicators such as CD31 and CD34. The mean time of observation was 871.6 days.

Results. Microscopic examination detected no malignant cells or micrometastases in the bone marrow in the analyzed group. Cytokeratin CAM 5.2 was detected in 33 cases (94.23%) in a lung tumour and in 21 cases (60%) in the bone marrow. Statistical analysis (chi² NW), showed a statistically significant relationship between the presence of CAM 5.2 expression in a tumour and in the bone marrow. In all analysed cases, the expression of cytokeratin AE1/AE2 was found in a tumour, but was not detected in any bone marrow sample. Cytokeratin CK-7 and CK-18 were present in a tumour in 20 (57.14%) and 23 (65.71%) patients, respectively. In the bone marrow, the expression of cytokeratin CK-7 was found in one case (2.86%), and CK-18 was not found in any patients. Thirteen (37.14%) patients died during follow-up. Local recurrence was diagnosed in three patients (8.57%) and distant metastases in 15 patients (42.86%). Mean recurrence-free time was 687.7 days.

Conclusions. On the basis of immunohistological tests, it was shown that a significant correlation existed between the presence of cytokeratin CAM 5.2 expression in a tumour and in the bone marrow. Its presence in the bone marrow was a good predictive factor for recurrence-free time. Mortality and the frequency of locoregional recurrence and distant metastases depend on pathological lung cancer staging.

Key words: lung cancer, micrometastases, immunohistochemistry, bone marrow, long-term survival
One of the sites of localization, besides regional lymph nodes, where micrometastases can be found is the bone marrow. The choice of bone marrow is supported by the fact that even cells of malignant neoplasms that generally do not metastasize to bones (e.g. colonic cancer) can be detected there (12). A question arises as to whether the detection of micrometastases in the bone marrow, even in patients with an early stage of cancer, may necessitate change in the planned treatment and casts doubt on the merit of surgical management. Does such a situation suggest that malignancy is already disseminated?

It is of great importance to distinguish micrometastases from circulating tumour cells. Micrometastasis is defined as a group of malignant cells whose longest diameter is equal to or smaller than 0.2 cm. These cells should not only be in direct contact with vessel walls or lymphatic sinuses, but also invade them, proliferate, and produce a stromal reaction (13), while circulating tumour cells refers to any detectable tumour cells in peripheral blood (12).

Microscopic cytological examination with routine hematoxylin-eosin staining is a dated methodology, but is still a useful diagnostic method for cancer detection. In the early stages of lung cancer, the concentration of tumour cells in the bone marrow is low and their detection by routine imaging techniques, biochemical tests, and microscopic examination is difficult or impossible. Therefore, more advanced techniques such as immunohistochemical and molecular methods are presently used to detect malignant cells in the medulla (5, 10, 14). Several different antibodies can be used to detect metastases in the bone marrow or lymph nodes. Their great advantage is the fact that they are epithelium-specific and show no reaction with normal lymphoid cells present in the medulla and lymph nodes. None of the antibodies, unfortunately, is specific for cancer and they react both with normal and malignant epithelial cells. However, they can be useful for the detection of epithelial cells in the bone marrow or lymph nodes where epithelial elements are normally not present. The reported sensitivity of immunohistochemical methods range from one cell in $10^5$ to $2-5$ in $10^6$ normal hematopoietic cells (5, 10, 14).

The objective of the study was to evaluate the usefulness of microscopic examination and immunohistochemical methods for the detection of micrometastases or cancer cells in the bone marrow of patients operated on for non-small cell lung cancer (NSCLC), as well as to assess long-term survival and disease free intervals dependent on the presence of cancer metastases in the bone marrow detected by immunohistochemical methods.

**MATERIAL AND METHODS**

Thirty-five patients (6 females and 29 males) from 47 to 78 years old (mean age 61.63 ± 8.75 years) operated on for NSCLC in 2003 were analyzed. The bone marrow was sampled from a broken or intentionally cut rib. Resected lung parenchyma along with a tumour and a bone marrow sample were placed on wet blotting paper and was sent to the Department of Pathology. Both samples obtained from a tumour and the bone marrow were stained using the same methods: routine hematoxylin-eosin staining and staining by immunohistochemical methods with antibodies AE1/AE3, CAM 5.2, CK-7 and CK-18 and vascular growth factors CD31 and CD34. Lung tumours were analyzed for their histopathological type, grade and the microscopic completeness of resection. In the immunohistochemical examination, a color reaction was assessed. A result was regarded as positive (positive color reaction) if more than 10% of the cells were stained. Within a tumour, only malignant cells were taken into account and both stromal and vascular stroma cells were excluded in contrary to bone marrow specimens where all cells were taken into account. All pathologic specimens were evaluated under a light microscope with magnification of 100x, 200x and 400x.

All patients were followed up in our outpatient clinic one month after surgery then every three months during the first year postoperatively and every six months thereafter. The follow-up was from 79 to 1319 days (average – 871.6 days). Recurrence free time, the number of deaths and their causes were analyzed.

To perform this study, we obtained permission from the Bioethical Committee of Collegium Medicum in Bydgoszcz, Mikolaj Koper- nik University in Toruń.

In evaluating statistical correlations between the presence of cytokeratins AE1/AE3, CK-18, CD31, and CD34 within a tumour and their presence in the bone marrow, no correlation tests could be used because one, possibly two variables couldn’t be divided into categories. The-
Therefore, statistical analysis was performed on the correlation of the presence of CAM 5.2 and CK-7 within a tumour and the bone marrow. The chi² test and contingency coefficient were used in statistical analysis as they enable the assessment of correlation between two variables that have any quantitative or qualitative character. Pearson’s method was also used for the analyzed group. To evaluate follow-up data log-rank, Cox-Mantel and F Cox tests were used.

RESULTS

Twenty-three lobectomies and twelve pneumonectomies were performed in the analyzed group. All patients underwent mediastinal lymph node dissection. On the basis of pathological examination, 25 cases of squamous cell carcinoma (71.43%), eight adenocarcinomas (22.86%), and two cases of large-cell carcinoma (5.71%) were found. Grading of the tumours was as follows: G1 – one case (2.86%), G2 – 24 cases (68.57%) and G3 – 10 cases (28.57%). Postoperative staging of NSCLC was as follows: IA – 6 (17.14%), IB – 15 (42.86%), IIA – 2 (5.71%), IIB – 5 (14.29%), IIIA – 7 (20%) cases. All surgical procedures were radical and this was proven by microscopic examination.

Routine histopathological examination detected neither micrometastases nor cancer cells in the bone marrow. In all 35 cases, cytokératin AE1/AE3 was found within a tumour, but in no case was it detected in the bone marrow. Cytokeratin CK-7 expression was detected in a tumour in 20 (57.14%) patients and CK-18 in 23 (65.71%) patients. Cytokeratin CK-7 was found in the bone marrow in one case (2.86%) and CK-18 was absent in all cases. Cytokeratin CAM 5.2 was detected within a lung tumour in 33 cases (94.23%) and in the bone marrow in 21 cases (60%).

CD31 and CD34 growth factors were found in no patient within a lung tumour. On the other hand, in the bone marrow, CD31 was present in 28 patients (80%), and CD34 in 32 patients (91.43%).

Statistical analysis with Chi² NW showed statistically significant correlation between the presence of CAM 5.2 in a lung tumour and the bone marrow (p=0.0498). In 64% of patients in whom cytokeratin CAM 5.2 was detected in a tumour, it was also present in the bone marrow, while it was not present in the bone marrow in any case where it was not detected in a tumour. No statistically significant correlation was found between the presence of cytokeratin CAM 5.2 and CK-7 in a tumour and the bone marrow and sex, age, histopathological type of lung cancer, its grade, or staging.

In patients in whom cytokeratin CK-7 was not detected within a tumour, it was also not present in the bone marrow. If cytokeratin CK-7 was found within a tumour, it was present in the bone marrow in only 5% of cases. The correlations were statistically insignificant.

On the basis of immunohistochemical examination, it was found that cytokeratin CAM 5.2 is useful for the detection of epithelial cells in the bone marrow in patients with NSCLC.

Follow-up in the outpatient clinic and the assessment of recurrence-free time in patients enabled the division of the analyzed patients into two study groups. The first group (I) consisted of patients with a recurrence of lung cancer, n = 18, and the second (II) consisted of patients in whom no recurrence was observed, n = 17. For both groups, recurrence-free time, pathological staging, and the presence of cytokeratins CAM 5.2 and CK-7 within a tumour and in the bone marrow were analyzed.

During the follow-up period, 13 of 35 patients died (37.14%), with death occurring 79 to 896 days after surgery (mean survival 436.92 days). Eleven patients died in group I and two patients died in group II. Pathological staging in these patients was as follows: IA – one case, IB – three cases, IIB – four cases, and IIIA – five cases. Of this group, in two patients, no local recurrence or distal metastases were found.

In 18 out of 35 patients (51.43%) (group I) in whom recurrence was observed, in three cases (8.57%) it was local recurrence and in 15 cases (42.86%) distant metastases were found: bones – three cases, brain – five cases, lung – six cases, cervical lymph nodes – one case. Recurrence-free time ranged from 73 to 1263 days (mean: 687.7 days). Patients with local recurrence had pathological lung cancer staging as follows: IB, IIB and IIIA, with one case in each stage. In all of these patients, cytokeratin CAM 5.2 was detected within a tumour, but was not present in the bone marrow. Cytokeratin CK-7 was found within a tumour in one case, but was not detected in the bone marrow in any of these patients. Pathological staging in patients with distant metastases was as follows: IA – one case, IB – six cases, IIA – two cases, IIB – two cases and IIIA – four cases. Cytokeratin
CAM 5.2 was detected in all 15 cases within the tumour, and in 11 cases in the bone marrow. Cytokeratin CK-7 was present within the tumour in eight patients, and in the bone marrow in only one patient.

In group II (17 patients – 48.57%), two patients died and no recurrence was diagnosed. Fifteen patients are still alive and have no recurrence. Recurrence-free time in this group ranged from 887 to 1263 days (mean: 1961.9 days). Pathological staging in the patients was as follows: IA – five cases, IB – eight cases, IIB – two cases and IIIA – two cases. In 15 patients, cytokeratin was detected within the tumour and in 10 cases in the bone marrow. Cytokeratin CK-7 was present within the tumour in 11 patients, whereas it was absent in the bone marrow in all cases.

Statistical analysis showed that the probability of survival in patients with lower pathological staging was higher than those with a higher pathological staging. The probability was higher in patients with stage IA than with stage IIIA (p=0.01752), in patients with stage IB than in patients with stage IIIA (p=0.02792) and in patients with stage IA and IB than in patients with stage IIIA (p=0.01248).

A negative correlation was found between the presence of cytokeratin CAM 5.2 in the bone marrow and the probability of survival. Recurrence-free time in patients in whom cytokeratin CAM 5.2 was present in the bone marrow was longer than in patients in whom it was absent (p=0.02403).

DISCUSSION

Surgery remains the gold standard treatment for locally advanced NSCLC. Early diagnosis of dissemination of lung cancer makes it possible to avoid unnecessary surgical treatment and enables the choice of adequate oncological therapy. Modern imaging techniques such as ultrasound, computed tomography, magnetic resonance imaging and positron emission tomography (PET) can detect pathological lesions larger than 1 cm³. Detection of metastatic sites that are clusters of less than 10⁹ malignant cells by those imaging techniques is impossible. In recent years, many immunological and molecular techniques were introduced that have enabled progress in the detection and characterization of occult, disseminated groups of malignant cells (4, 5, 6, 12, 14).

Immunohistochemical examination makes it possible to detect indirectly micrometastases of the colon, prostate, breast and lung in bone marrow. Micrometastases are detected in the bone marrow in 20-40% of patients with operable breast cancer and in 20-70% of patients with distant metastases (5). Recent reports analyze the presence of micrometastases in the bone marrow in patients with operable NSCLC (1, 3-6, 9-12). Pantel et al. investigated the bone marrow from iliac crest aspirate in 82 patients and in 22% of the cases malignant cells were detected by immunohistochemical techniques. They proved that the detection of cancer cells in the bone marrow was significantly correlated with tumour size and grading (6). Jiao et al. found a higher rate of detected cancer cells in the bone marrow obtained from rib segment resection than iliac crest aspirate (12). Other authors such as Cote et al. used immunohistochemical techniques to detect malignant cells in the bone marrow in 17 out of 43 patients with early stage cancer that had no clinical manifestations of disseminated malignant disease (5). They used in their study two monoclonal cytokeratin-specific antibodies AE-1 and CAM 5.2. The presence of malignant cells in the bone marrow was significantly correlated with cancer staging. They were detected in 29% of patients with stage I and II and in 46% of patients with stage III.

In our study, neither micrometastases nor single tumour cells were detected in the bone marrow by routine histopathological examination in any case. On the other hand, immunohistochemical examination for cytokeratin CAM 5.2 detected the presence of epithelial cells in the bone marrow in as many as 60% of patients. Furthermore, the correlation between the presence of CAM 5.2 in a tumour and in the bone marrow in the patients was statistically significant. No statistically significant correlation was found between the presence of cytokeratin CAM 5.2 in the bone marrow and sex, age, histopathological type of lung cancer, its grade, or staging.

Cote et al. noted also that local recurrence was observed earlier in patients with immunohistochemical features of micrometastases of NSCLC in the bone marrow. The median time to recurrence for patients with no detectable occult metastases was 35.1 months compared with 7.3 months for patients with occult
metastases. Overall survival was also lower in patients with occult micrometastases in the bone marrow compared with patients without occult metastases, although patients in both groups had similar lung cancer stage and underwent similar treatment (5).

In our study, we found that the overall survival and recurrence-free time were correlated significantly only with clinical cancer stage. It was also found that the presence of cytokeratin CAM 5.2 in the bone marrow had a positive influence on prognosis, which is an unexpected finding and quite different from results obtained by others.

Other authors are of the opinion that studies for the detection of NSCLC micrometastases in the bone marrow should be continued as they could be a basis for the modification of the present TNM staging system of NSCLC and could lead to more precise qualification of patients to combined treatment (chemotherapy + surgery), even in patients with early stage NSCLC.

**CONCLUSIONS**

1. On the basis of immunohistochemical examination, it was found that a significant correlation exists between the presence of cytokeratin CAM 5.2 in the tumour and in the bone marrow in patients operated on for NSCLC. The detection of cytokeratin CAM 5.2 is a good prognostic factor in relation to recurrence-free time.

2. There was no statistically significant correlation between the presence of immunohistochemical features of micrometastases in the bone marrow and sex, age, histopathological type of lung cancer, its grade, or staging.


4. Cancer-related mortality along with the frequency of local recurrence and distal metastases depends on pathological cancer stage.

**REFERENCES**


Received: 12.09.2007 r.
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