Cytokeratin 19 mRNA Concentration in Lymph Nodes as a Diagnostic Marker of Metastases

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Comparison of the sensitivity of cytological and molecular genetic methods (19 specimens of lymph nodes from 8 patients with breast cancer and suspected metastases obtained by transcutaneous fine-needle aspiration biopsy under ultrasound guidance) showed that molecular genetic and cytological studies produced true results in 95 and 84% specimens, respectively. True-positive and true-negative results were obtained in 8 and 7 patients, respectively. Expression of cytokeratin 19 was detected in 3 specimens with negative cytological results and confirmed metastases in lymph nodes. Our results indicate that molecular genetic diagnostic study for lymph node metastases should be used in small amounts of biopsy specimens, presence of marginal metastases in lymph nodes, and negative results of repeated cytological examination.

Key Words: *cytokeratin 19; lymph nodes; breast cancer*

Clinically verified metastases and micrometastases are found in 30 and 30-35% patients with detectable primary breast cancer (BC), respectively [5]. The involvement of regional lymph nodes in the tumor process has a negative effect on the 5-year survival rate. In many patients, metastases develop after radical surgery, which is related to the presence of undetected tumor cells in the lymph nodes.

Complex clinical X-ray and ultrasound examination is used for the diagnostics of metastases in regional lymph nodes. This approach reveals the pathological process in 70-95% patients. However, there are significant difficulties in the differential diagnostics. Cytological examination is a simple and relatively inexpensive method. However, the accuracy of this study depends on the conditions for isolation and treatment of the biopsy specimen. The reliability of cytological diagnostics for BC is 70-85%. Puncture is uninformative in 25-30% patients. Hence, the search for new objective methods of the study is an urgent problem. Molecular genetic methods are sensitive and allow us to identify single cancer cells among 10^4-10^5 nontumor cells in the lymph node. PCR methods for the detection of metastases are characterized by higher sensitivity. There are data on the detection of mRNA for the following markers: CEA (cancer embryonic antigen), CK-19 (cytokeratin 19), CK-20 (cytokeratin 20), mammaglobin, and MUC-1. Comparative study showed that PCR diagnostics is more informative than histological methods. The 4-year survival rates of patients with PCR and histologically verified metastases are 100 and 74%, respectively (p=0.03) [7].

Here we compared the sensitivity of cytological and molecular genetic methods for the diagnostics of lymph node metastases in BC.

MATERIALS AND METHODS

Lymph node specimens were obtained from 11 patients with BC and suspected metastases and 8

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patients with reactive changes in lymph nodes. The patients were subjected to clinical X-ray and ultrasound examination. The specimens were obtained by means of transcutaneous fine-needle aspiration biopsy under ultrasound guidance. Voluntary informed consent was obtained from all patients.

CK-19 mRNA expression was used as a marker of cancer cells. CK-19 mRNA concentration was measured by means of quantitative real-time PCR. All specimens were subjected to cytological analysis. Panchromatic azure-eosin staining by the method of Romanovsky—Giemsa in modifications of Leishman and Pappenheim was used. The results of cytological and molecular genetic studies were compared with planned histological examination of tissues after mastectomy and radical lymph dissection.

Total RNA was isolated using PNeasy Plus mini kit (Qiagen) according to manufacturer's recommendations. RNase inhibitor was added to the aqueous solution of RNA (35-55 μl) to a final concentration of 1 U/ml. RNA solution was stored at -70°C.

The reverse transcription reaction was performed with random sequence hexamer primers and reverse transcriptase MMLV (Sileks).

The initial solution of RNA (2 μl), random hexaprimers (15 optical U/ml, 1 μl), RNase inhibitor (1 μl), and deionized water (14 μl) were mixed in 600-μl PCR tubes. The mixture was incubated at 70°C for 5 min and put on ice; 2.5 μl 10-fold reverse transcription buffer, 4 μl 1.5 mM dNTP mixture, and 0.5 μl reverse transcriptase MMLV (20 U/μl) were added. The mixture was incubated according to the following scheme: 10 min at 25°C, 60 min at 37°C, and 10 min at 70°C. Complementary DNA (cDNA) was cooled on ice and used for real-time PCR.

All procedures of reverse transcription except for addition of reverse transcriptase MMLV were performed to evaluate the presence of DNA in the specimen.

The concentration of CK-19 mRNA in specimen was measured by real-time PCR. Measuring the concentration of hypoxanthine-guanine phosphoribosyltransferase (HPRT1) gene mRNA served as an internal standard. The study was conducted with primers for HPRT1 (forward, 5'-AAGCCAGA CTTTGTTGGATTTGA-3'; reverse, 5'-AACTTGAA CTCTCATCTTAGGCTTT-3'; specimen, 5'-FAMTT GTTGTAGGATATGCCCTTGACTATAATGAAB

TABLE 1. Results of Cytological and Histological Studies and Concentration of CK-19 mRNA in Biopsy Specimens from the Lymph Node of BC Patients

Number of specimen	Cytological study	CK-19 mRNA/HPRT1 mRNA	Histological study
1	MTS	655.6885	MTS
2	MTS	64.8623	MTS
3	MTS	1.161912	MTS
4	No	0	No
5	No	476.8749	MTS
6	No	0	No
7	No	10 735.26	MTS
8	No	0	No
9	No	0.159499	No
10	No	116.4697	MTS
11	MTS	217.797	MTS
12	MTS	1995.105	MTS
13	MTS	1798.449	MTS
14	No	0	No
15	No	—	No
16	No	0	No
17	MTS	68.47784	MTS
18	No	0	No
19	MTS	72.83869	MTS

Note. MTS: metastasis.

HQ1-3') and CK-19 (forward, 5'-CCA GCC GCT CAT GGA CAT-3'; reverse, 5'-CTT GGA GGC AGA CAA ATT GTT G-3'; specimen, 5'-FAM AGC AGG AGA TTG CCA CCT ACC GCA BHQ1-3').

Amplification was performed in 3 repetitions to increase reliability of the results. The reaction mixture (final volume 20 μl) contained 0.3 mM forward and reverse primers, 0.25 mM specimen, 1.2 mM $MgCl_2$, 0.2 mM dNTP mixture, 1x buffer for taq polymerase, and 1 U Hot Start Taq DNA polymerase. Real-time PCR was performed on an ANK-32K-4Ts device (Institute of Analytical Instrument-Making, Russian Academy of Sciences, Russia). Taq polymerase activation and DNA melting were performed at 95°C for 900 sec (1 cycle). Annealing of primers and elongation of DNA chains were performed at 60°C for 60 sec, and melting at 95°C for 15 sec (40 cycles).

The critical cycle and initial content of DNA copies were estimated by means of ANK-32 software.

The mean number of genome CK-19 DNA copies was subtracted from the total number of CK-19 copies in the specimen to study CK-19 mRNA expression.

The relative content of CK-19 mRNA in the specimen was estimated from the concentration of mRNA for reference gene HPRT1 in the same specimen. This gene is characterized by stable and low expression of mRNA in cells. We calculated the ratio of CK-19 gene expression to internal reference gene (HPRT1) expression.

The results were analyzed statistically by means of Statistica 6.0 software.

RESULTS

One of 19 biopsy specimens from lymph nodes was uninformative. CK-19 mRNA was detected in 11 specimens (Table 1).

The CK-19 mRNA/HPRT1 mRNA ratio <1 indicated the absence of lymph node metastases. Increased expression of CK-19 mRNA indicated the presence of metastases from BC.

The results of molecular genetic and cytological studies were consistent in 15 specimens (83.3%). True-positive results (presence of cancer cells and CK-19 expression) were detected in 8 patients. True-negative results (cytologically undetected cancer cells and no expression of CK-19) were obtained in 7 patients.

CK-19 expression was revealed in 3 specimens from 2 patients with negative cytological results and histologically confirmed metastases in lymph nodes (Table 1). The axillary lymph node in patient S. with suspected metastases (clinical X-ray examination) was punctured 2 times due to negative cytological results. CK-19 expression was high in both specimens (specimens 5 and 7), which indicated the presence of metastases. These results were confirmed histologically.

Expression of CK-19 and HPRT1 was low in specimen 15. Hence, this specimen was of low quality due to long-tern storage (>1 month) or inadequate storage conditions.

Therefore, the molecular genetic and cytological studies produced true results in 95 and 84% specimens, respectively. However, complex clinical X-ray and ultrasound examination was informative in only 65.3% patients with marginal metastases.

We conclude that CK-19 expression in lymph node specimens can be used as a diagnostic marker of cancer cells.

Molecular genetic diagnostic study for lymph node metastases should be used in small amounts of biopsy specimens, presence of marginal metastases in lymph nodes, and negative results of repeated cytological examination.

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