PROGNOSTIC RELEVANCE OF OCCULT TUMOR CELLS IN LYMPH NODES IN COLORECTAL CANCER

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ABSTRACT

**Background:** Presently, in Europe the treatment of node-negative colorectal cancer (CRC) patients consists of surgical resection of the primary tumor without adjuvant systemic therapy. However, up to 30% of these patients will develop disease recurrence. These high-risk patients are possibly identified by occult tumor cell (OTC) assessment in lymph nodes. In this paper, studies on the clinical relevance of OTC in lymph nodes are reviewed.

**Methods:** A literature search was conducted in the National Library of Medicine by using the keywords colonic, rectal, colorectal, neoplasm, adenocarcinoma, cancer, lymph node, polymerase chain reaction, mRNA, immunohistochemistry, micrometastases and isolated tumor cells. Additional articles were identified by cross-referencing from papers retrieved in the initial search.

**Results:** The upstaging percentages through OTC assessment and the prognostic relevance of OTC in lymph nodes vary among studies, which is related to differences in techniques used to detect OTC.

**Conclusions:** We conclude that OTC examination techniques should be standardized to illuminate whether OTC in lymph nodes can reliably identify high-risk node-negative patients.

INTRODUCTION

High-risk node-negative colorectal cancer (CRC) patients may be identifiable through the detection of occult tumor cells (OTC) in lymph nodes. OTC comprise micrometastases (MM) and isolated tumor cells (ITC). MM are defined as deposits of tumor cells of 2 mm or less but larger than 0.2 mm and ITC either as single tumor cells or as clusters of tumor cells of 0.2 mm or less.\(^1\)\(^-\)\(^3\) OTC are usually not detected with conventional pathological examination, as only one or two 4 to 5 μm sections of each lymph node are being examined after staining with the hematoxylin and eosin (HE) method. It is calculated that a single 4 μm section through the center of a lymph node measuring 1 cm in diameter merely samples approximately 0.06 percent of the lymph node\(^4\) which is presumed to reflect the entire lymph node. Examination techniques focused on detection of OTC include serial sectioning, step sectioning, immunohistochemistry (IHC), polymerase chain reaction (PCR) and reverse transcriptase polymerase chain reaction (RT-PCR). There is a higher chance of detecting OTC with these techniques as a larger part of the lymph node is being examined and because of their higher sensitivity for detecting tumor cells.
The role of OTC detection in CRC is not clear yet although numerous studies on this topic have previously been published. This review deals with the detection methods and clinical relevance of OTC in lymph nodes in CRC. Emphasis is put on differences in examination techniques to detect OTC.

**METHODS**
A literature search was conducted with PubMed software in the National Library of Medicine, containing articles from 1953 until 2004. The following key words were used in appropriate combinations: colonic or rectal or colorectal neoplasm, adenocarcinoma and cancer, lymph node, polymerase chain reaction, mRNA, immunohistochemistry, micrometastases and isolated tumor cells. Papers with anal cancer in the title were excluded and the language was restricted to English. All hits from this PubMed search were individually checked, and included only if they addressed the subject of this review. Additional articles were identified by cross-referencing from papers retrieved in the initial search. Overall, only articles that included the prognostic relevance of OTC in lymph nodes were included for this overview. There was no limit to the number of patients.

**Occult tumor cells in lymph nodes detected with polymerase chain reaction**
The PCR assay can be used to detect OTC in lymph nodes by amplification of certain DNA regions that may contain tumor-specific mutations for instance regions in the tumor suppressor genes p53 and K-ras. The K-ras gene can contain mutations at codon 12, 13 or 61 and in the p53 gene mutations cluster in exons 5 to 8. There are four published studies in which HE-negative lymph nodes were examined with the PCR method and the effect of a positive PCR on the prognosis of the patients was analyzed. Hayashi et al. screened HE-negative lymph nodes for the presence of mutations in the K-ras or p53 genes. Twenty-seven out of 37 patients with PCR positive lymph nodes suffered from tumor recurrence within 5 years, whereas none of the 34 patients with OTC-negative lymph nodes had a recurrence. Thebo et al. used K-ras mutations at codon 12 and 13 to detect OTC. No recurrences were reported in the OTC-negative group (n = 4) compared to a recurrence rate of 38% in the OTC-positive group (n = 16). Clarke et al. examined lymph nodes by using K-ras mutations at codon 12. No difference was shown in survival between patients with a positive PCR and patients with a negative PCR. Four out of 13 OTC-positive patients died of recurrence within 5 years and one out of four OTC-negative patients also died of recurrence. Belly et al. also used K-ras mutations at codon 12 to detect OTC. Of 14 OTC-positive patients, eight died of disease within 5 years compared to four out of 24 OTC-negative patients. A disadvantage of the PCR method is the fact that the p53 and K-ras mutations do not occur consistently in CRC. Mutations of the p53 gene and the K-ras gene are present in approximately 70% and 38% in CRC, respectively. Only when
the primary tumors harbor mutations, these mutations can be utilized to detect OTC in HE-negative lymph nodes. Hayashi et al. could not detect mutations in the p53 or K-ras gene in 41% of 120 primary tumors leading to the exclusion of many patients for OTC detection. An additional problem is the large number of codons or even exons in which mutations can be detected, especially in the p53 gene. This implies that for detection of all the possible mutations many different PCR primers are needed which is not feasible in clinical practice and rather expensive. Concluding, three out of four studies showed clinical relevance of OTC detected with the PCR method. However, technical factors will prevent the PCR from becoming a ubiquitously utilized application for OTC detection.

Occult tumor cells in lymph nodes detected with reverse transcriptase polymerase chain reaction

RT-PCR within the context of OTC assessment in CRC involves amplification of specific messenger RNA present in epithelial cells or malignant cells. A study by Liefers et al. showed that patients with CEA RT-PCR negative lymph nodes had a significantly better 5-year disease-free survival than patients with positive lymph nodes (91 vs 50%, P = 0.02). Five other RT-PCR studies also showed that a positive RT-PCR result had a negative impact on survival. Three of these 6 studies used CEA, 2 used CK20 and 1 used GCC as a marker. One of these studies also used CEA as a marker and showed no difference between patients with a positive RT-PCR result and patients with a negative result. However, with this marker they showed an upstaging percentage of 5 whereas the other three CEA studies showed an upstaging range of 30% to 54%. This suggest a limited sensitivity of this specific protocol what may explain the lack of influence on clinical outcome. The low upstaging percentage of 5% could be due to the use of paraffin-embedded lymph nodes.

During the past years a one-tube one-enzyme quantitative real-time RT-PCR has been developed by several companies. This method involves a closed system and makes use of fluorescence to quantify the PCR product. Real-time RT-PCR is highly sensitive, has a lower risk of contamination and is far less laborious compared to the conventional RT-PCR. Lassmann et al. showed no prognostic value of a positive real-time RT-PCR test with the marker CK20. A recent study by Bustin et al. neither found any correlation between patient’s prognosis and a positive real-time RT-PCR test when using the markers CK20, CEA and GCC at 40 PCR cycles. The higher sensitivity of the real-time RT-PCR is shown in the upstaging percentages with the markers CEA and CK20 of 98% and 75%, respectively. The reason why Lassmann et al. found a lower upstaging percentage of 35% with their real-time CK20 RT-PCR might relate to the use of paraffin material. Furthermore, the lower GCC upstaging percentage of 22% in the study by Bustin et al. compared to the GCC upstaging percentage of 48% in the study by Cagir et al. could be the result of a higher number of PCR cycles in the latter study i.e.
40 cycles versus 70 cycles. A disadvantage of the higher sensitivity of the real-time RT-PCR is the detection of background gene expression in hematopoietic cells or circulating normal epithelial cells, and thus false-positive patients, that might explain the higher percentage of upstaging when compared to the earlier studies. The rate of false-positive patients can be limited by optimizing the number of PCR cycles. Miyake et al. reported that under 35 cycles of PCR, lymph nodes from patients with benign diseases did not express CEA and CK20, but, when using 40 cycles, bands for CEA appeared in 7% of normal lymph nodes and bands for CK20 appeared in 20% of normal lymph nodes. These results indicate that there is only a narrow window in which reliable results can be obtained. Similar results were reported earlier by Liefers et al. who examined HE-negative lymph nodes by using a nested RT-PCR. A very faint band was detected in some negative control samples when 20 or 25 cycles were used in the second PCR round. Therefore, it was decided that 15 cycles should be used in the second PCR round leading to a total of 35 cycles in their nested RT-PCR method.

A pitfall associated with the PCR and RT-PCR method is contamination of lymph nodes by cells from the primary tumor or bowel epithelium leading to false-positive results. Rosenberg et al. examined HE-negative lymph nodes with both RT-PCR and IHC. In 13 of 44 RT-PCR positive patients, the positivity was caused by tumor cell contamination located exclusively outside the lymph node capsule. Defining these 13 patients as RT-PCR negative improved the specificity of the RT-PCR assay from 57% to 75%. The overall 5-year survival rates of the IHC-controlled RT-PCR positive group were significantly worse than the negative group (P < 0.001), and the differences were greater than with RT-PCR alone (P < 0.009). Contamination of lymph nodes by cells from the primary tumor or bowel epithelium can be limited by removing the lymph nodes before incision of the bowel specimen during examination by the pathologist.

From these data it can be concluded that OTC in lymph nodes detected with RT-PCR show prognostic value provided that fresh or frozen lymph nodes, the markers CEA, CK20 and GCC and the optimal number of PCR cycles are used.

**Occult tumor cells in lymph nodes detected with immunohistochemistry**

Although RT-PCR is potentially more sensitive than IHC, the latter method is commonly available in daily practice and has the advantage of morphological confirmation of detected tumor cells. Conversely, the specificity of IHC seems to be lower. Noura et al. studied paraffin-embedded lymph nodes with both CEA RT-PCR and IHC by using the anti-pancytokeratin antibody AE1/AE3 and showed that the former method had prognostic value, whereas the latter did not.

Nine out of 28 studies showed a significantly worse clinical outcome in patients with lymph nodes containing OTC: five out of eight studies using CAM5.2 (63%) out of seven using AE1/AE3 (14%) 15;21;32-36 out of two using CEA (50%) 28;37 out of two using CK20 (50%) 17;28 out of two using
of two using MNF116 (50%)\textsuperscript{24,38}, zero out of two using BerEP4 studies\textsuperscript{28,35}, zero out of one using CC49\textsuperscript{21}, zero out of one using anti-CK\textsuperscript{39}, zero out of one using KL-1\textsuperscript{40}, and zero out of one using RSP53 antibodies.\textsuperscript{40} One study, using a mixture of antibodies among which AE1/AE3 and CAM5.2, reported no worse prognosis of patients with OTC in lymph nodes.\textsuperscript{41} Three\textsuperscript{17,28,30} out of the 28 studies showed a trend towards a worse clinical outcome: one\textsuperscript{30} out of eight studies using the antibody CAM5.2 (13%), one\textsuperscript{17} out of two using CK20 (50%), and one\textsuperscript{28} of two using BerEP4 (50%) antibodies. The other 15 studies showed no prognostic effect of OTC-positive lymph nodes. Eight\textsuperscript{21-28} of 22 studies\textsuperscript{15,17,21-36,38-41} (36%) using antibodies directed against cytokeratin showed a worse prognosis of patients with OTC in lymph nodes compared to one\textsuperscript{28} of six studies\textsuperscript{21,28,35,37,40} (17%) using antibodies directed against other antigens.

In conclusion, only nine out of 28 IHC studies showed a significantly worse clinical outcome in patients with lymph nodes containing OTC. In particular studies using the antibody CAM5.2, reported OTC-positive lymph nodes to be of clinical relevance.

**Factors influencing detection of occult tumor cells with immunohistochemistry**

Xu et al.\textsuperscript{42} reported undesirable cytokeratin positivity in nonepithelial cells in lymph nodes from breast cancer patients. Cytokeratin positivity was found in reticulum cells and plasma cells with pan-CK and CAM5.2 but not with AE1/AE3\textsuperscript{a}. Seemingly, antibodies raised primarily against CK8 (CAM5.2 and pan-CK) can detect background CK8 expression in nonepithelial cells, which cannot be revealed by AE1/AE3, though it recognizes a broad spectrum of different cytokeratins. Adversely, use of a broad spectrum antibody such as AE1/AE3, might not be as sensitive as other subset-specific antibodies like CAM5.2 in identifying certain undifferentiated carcinomas. This might explain the higher percentage of CAM5.2 studies showing clinical relevance compared to AE1/AE3 studies as mentioned above. Morphological evaluation is key in distinguishing tumor cells from nonepithelial cells. Others methods that can be used to distinguish tumor cells from plasma cells, dendritic cells, mesothelial cells and macrophages, include double staining with anti-immunoglobulin kappa or lambda light chains, anti-S100, anti-calretinin, and anti-CD68 antibodies respectively. Studies comparing antibodies should lead to the antibody with the highest sensitivity and specificity in recognizing tumor cells in lymph nodes.

Three\textsuperscript{27,28} out of eight studies\textsuperscript{27,28,32,35,41} examining more than one level per lymph node showed prognostic significance of OTC compared to six\textsuperscript{21-26} out of 20 studies\textsuperscript{15,17,21-26,29-31,33,34,36-40} examining one level. Two\textsuperscript{22,25} out of six studies\textsuperscript{15,22,25,39,40} examining more than one section per level showed prognostic significance of OTC.

\textsuperscript{a} There are 20 different types of cytokeratins; CAM5.2 recognizes CK7 (weak) and 8; AE1/AE3 is a mixture of two different clones of monoclonal antibodies; AE1 recognizes CK10, 13, 14, 15, 16, and 19; AE3 recognizes CK1, 2, 3, 4, 5, 6, 7 and 8; MNF116 recognizes CK5, 6, 8, 17 and probably 19; anti-CK recognizes CK 8, 18 and 19; KL-1 recognizes CK1, 2, 5, 6, 7, 8, 11, 14, 16, 17 and 18.
compared to seven\textsuperscript{21;23;24;26-28} out of 22 studies\textsuperscript{17;21;23;24;26-38;41} examining one section per level. Adell \textit{et al.}\textsuperscript{39} reported that 95\% of CRC patients with OTC were identified by examining three levels from three lymph nodes with AE1/AE3. McGuckin \textit{et al.}\textsuperscript{43} examined lymph nodes from breast cancer patients and showed that the majority of lymph node metastases can be detected by examining two levels 300 \(\mu\)m apart. Fisher \textit{et al.}\textsuperscript{33} studied the largest CRC patient group thus far and found OTC in lymph nodes in 18.3\% of 399 patients but could not confirm any prognostic significance of OTC in lymph nodes. This may be related to the examination of only one level of each lymph node in combination with the use of the antibody AE1/AE3.

In all of the abovementioned papers, OTC were detected through screening by a pathologist using routine light microscopy. This bares the risk of inaccurate screening due to factors such as OTC size, interobserver differences and incomplete section screening. Automated microscopy may facilitate and render IHC more reliable because the aforementioned factors are eliminated.\textsuperscript{44;45} As yet, an optimum number of two levels has been reported. It can be suggested that in order to assess whether OTC in lymph nodes predicts patient’s prognosis, at least two levels of the lymph nodes should be examined using an anti-cytokeratin antibody.

\textbf{Number and size of lymph nodes}

In the studies by Sasaki \textit{et al.}\textsuperscript{22} and Yasuda \textit{et al.}\textsuperscript{25}, not only the presence but also the number of OTC-positive lymph nodes was considered in relation to decreased survival. The former study showed a significantly higher frequency of OTC-positive nodes in patients with recurrent disease. The latter study reported OTC in four or more lymph nodes to be significantly associated with recurrent disease. Both studies showed a significantly higher frequency of OTC in second tier lymph nodes of patients with poor prognosis.

Overall survival of CRC patients without HE detectable nodal metastases, improves with increasing number of lymph nodes recovered.\textsuperscript{46;47} Cserni \textit{et al.}\textsuperscript{47} studied data from 8574 stage II CRC patients. They could not define a cut-off value for the number of lymph nodes needed to be examined for adequate nodal staging. According to their statistical analysis the risk of death decreased by 2.1\% for each negative lymph node. Ruers \textit{et al.}\textsuperscript{48} reported the number of nodes that had to be examined for reliable staging to be T stage dependent. Fifteen nodes for T2 tumors, 10 nodes for T3 tumors and seven nodes for T4 tumors needed to be examined. Current guidelines from the American Joint Committee on Cancer Staging (AJCC) recommend examination of a minimum of 12 lymph nodes for accurate staging.\textsuperscript{49} However, this is not always feasible. By studying 569 CRC specimens, Johnson \textit{et al.}\textsuperscript{50} reported that only 22\% of the patients underwent an adequate lymph node harvest according to the current AJCC recommendation. Sometimes only few or no lymph nodes are found, even when surgeons resect a
large part of the perimuscular fatty tissue. Involved factors might be preoperative radiotherapy in rectal carcinoma, leading to decrease in lymph node size\textsuperscript{51}, or the presence of few or very small lymph nodes.\textsuperscript{52} The former factor was shown in the Total Mesorectal Excision trial where the mean number of examined lymph nodes was 9.7 in the surgery only group and 7.7 in the radiotherapy group (P < 0.001).\textsuperscript{51} Maurel \textit{et al.}\textsuperscript{52} reported a significantly higher number of examined lymph nodes in patients younger than 75. Fat clearance techniques might facilitate in finding small lymph nodes. Haboubi \textit{et al.}\textsuperscript{53} showed that by adding fat clearance to conventional lymph node harvesting, the mean number of recovered lymph nodes increased from 6.7 to 58.2 lymph nodes. However, these relatively inexpensive techniques involve the use of xylene and alcohol and, therefore, are considered impractical and unsafe. This is the major reason that fat clearance techniques are not being used worldwide. It should be noted that even very small lymph nodes can contain metastases. Andreola \textit{et al.}\textsuperscript{41} reported that 45\% of metastatic lymph nodes of 49 stage III patients had a diameter smaller than 5 mm, determining the stage in 15 (31\%) of the patients. Haboubi \textit{et al.}\textsuperscript{30} showed that 86\% of lymph nodes with OTC had a diameter smaller than 5 mm. Ruers \textit{et al.}\textsuperscript{48} suggested that a standard of how much cm\(^2\) of mesocolon should be removed by the surgeon and how many lymph nodes should be retrieved by the pathologist per cm\(^2\) will contribute to quality control in colon cancer.

Summarizing, the number of OTC-positive lymph nodes should also be considered in relation to decreased survival. Moreover, the problem of defining an optimal number of lymph nodes and finding small lymph nodes might be tackled by standardization of how much cm\(^2\) of mesocolon should be removed by the surgeon.

**Size and location of occult tumor cells in lymph nodes**

Using the actual size of OTC instead of dividing them into categories such as ITC and MM might answer the question whether the size of metastases matters.\textsuperscript{33} As yet it is not clear whether MM and ITC have the same influence on the patient’s clinical outcome. It is possible that in immunocompetent individuals, OTC are destroyed by the immune system before growing into large metastases or that not all OTC are viable and therefore do not have the capacity to proliferate. Determining the viability of OTC could shed some light on the metastasis potential of these tumor cells e.g. by determining the number of apoptotic cells \textit{versus} vital cells. Furthermore, it may be important to consider the intra-nodal location of OTC in the analysis. Tumor cells in lymph nodes can be located in subcapsular sinuses, also referred to as peripheral sinuses, paracortical sinuses or medullar structures. They can also be located intrafollicularly or show a diffuse distribution. It has been shown that tumor cells are usually present in the subcapular sinuses\textsuperscript{29,32,37,54,55} but the impact of the location of OTC on patient’s prognosis has not been clearly addressed yet. In summary, when considering OTC in lymph nodes in CRC, it might be necessary to include the size and intra-nodal location of OTC in the analysis.
CONCLUSIONS
Most of the studies indicate a clinical significance of detecting OTC in lymph nodes of CRC patients. Conditions and techniques, however, vary considerably among the different studies. Therefore, OTC examination should be standardized. We recommend comparing studies using RT-PCR and IHC to establish optimal conditions to reliably identify high-risk patients that are, using the current techniques, considered as lymph node-tumor negative patients.

REFERENCES


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