

Detection of Lymph Node Micrometastases by Gene Promoter Hypermethylation in Samples Obtained by Endosonography-Guided Fine-Needle Aspiration Biopsy

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ABSTRACT

Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) has become a fundamental procedure for gastrointestinal and lung cancer staging. However, there is growing evidence that micrometastases are present in lymph nodes, which cannot be detected with standard pathological methods. The aim of this study was to evaluate whether hypermethylation gene promoter analysis was feasible on samples obtained by EUS-FNA from lymph nodes, as well as to establish the usefulness of this strategy for the detection of micrometastases in patients with gastrointestinal and non-small cell lung cancer.

Suspicious lymph nodes based on EUS findings from consecutive patients with esophageal, gastric, rectal, and non-small cell lung cancer were sampled by EUS-FNA. Hypermethylation analysis of the *MGMT*, *p16^{INK4a}*, and *p14^{ARF}* gene promoter CpG islands were performed by methylation-specific PCR. Effectiveness of conventional cytology, methylation analysis, and their combination were established with respect to the definitive diagnosis.

Twenty-seven patients were included, thus representing a total of 42 lymph nodes (esophageal cancer, $n = 11$; rectal

cancer, $n = 7$; gastric cancer, $n = 3$; and lung cancer, $n = 21$). According to definitive diagnosis, 21 (50%) corresponded to metastatic lymph nodes. Sensitivity, specificity, and overall accuracy of conventional cytology were 76%, 100%, and 88%, respectively, whereas the corresponding values for the methylation analysis were 81%, 67%, and 74%, respectively. Combination of both techniques increased sensitivity (90%) but decreased specificity (67%) with respect to conventional cytology.

In conclusion, it is feasible to detect occult neoplastic cells in EUS-FNA samples by hypermethylation gene promoter analysis. Moreover, addition of methylation analysis to conventional cytology may increase its sensitivity at the expenses of a decrease in its specificity.

INTRODUCTION

Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) has become a well-established procedure for diagnosing and staging both gastrointestinal and lung cancer. Its high sensitivity and accuracy for the detection of regional metastatic lymph nodes has had a major impact on preoperative treatment strategies of these neoplasms, and it seems to be a more cost-effective approach than other staging modalities (1–5).

There is growing evidence that early micrometastases are present in lymph nodes, which cannot be detected with standard cytological or histological methods. The recent introduction of molecular techniques, such as PCR and reverse transcription-PCR, has shown to improve the detection of metastatic epithelial cells in lymph nodes, bone marrow, and peripheral blood from patients with lung, pancreatic, and gastrointestinal neoplasms (6–9). In lung cancer, up to 50% of patients with histologically normal lymph nodes have micrometastases detected by molecular techniques, their presence having a significant impact on patient long-term survival (10–16).

Molecular detection of micrometastases in lymph nodes from patients with epithelial malignancy has been attempted by using several approaches. Overall, these strategies encompass targeting both tumor-specific alterations (*i.e.*, DNA mutations in genes involved in its pathogenesis such as *KRAS* in pancreatic cancer or *TP53* in colorectal cancer; Refs. 9, 13) or tissue-specific changes (*i.e.*, RNA expression of genes differentially expressed, such as cytokeratins or carcinoembryonic antigen; Ref. 6). However, a common limitation of these approaches is the lack of high-sensitive, high-specific molecular markers for each type of neoplasm. Aberrant methylation of CpG islands in the promoter region of tumor suppressor genes has been recognized recently as an important epigenetic mechanism for gene silencing (17, 18). Inactivation of multiple tumor suppressor genes by aberrant hypermethylation is a fundamental process involved in the development of many malignant tumors, includ-

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ing gastrointestinal and lung cancer (17, 19–23). Of note, a unique profile of promoter hypermethylation exists for each human cancer in which some gene changes are shared and others are cancer type-specific (22). In that sense, gastrointestinal tumors share a set of genes undergoing hypermethylation including *p16^{INK4a}*, *p14^{ARF}*, *MGMT*, *APC*, and *hMLH1*, whereas lung cancer has a different pattern involving *DAPK*, *MGMT*, and *p16^{INK4a}* genes (22, 23). This spectrum of epigenetic alterations for a relatively small subset of genes constitutes a potentially powerful system of highly specific biomarkers.

The aim of present study was to evaluate whether hypermethylation gene promoter analysis was feasible on samples obtained by EUS-FNA from lymph nodes, as well as to establish the usefulness of this strategy for the detection of micrometastases in patients with gastrointestinal and non-small cell lung cancer.

PATIENTS AND METHODS

Patients. Between September 2001 and July 2002, all of the consecutive patients with esophageal, gastric, rectal, and non-small cell lung cancer and no proven distant metastases referred for EUS staging were considered. Patients were included in the study if they had any lymph node showing EUS features of malignancy (size >1 cm, round shape, sharp margin, or diffusely hypoechoic). Exclusion criterion was refusal to participate in the study and coagulopathy. The institutional Ethics of Research Committee approved the protocol, and informed consent was obtained from each patient.

EUS-FNA was carried out as part of the diagnostic protocol of our Unit. This procedure was performed under conscious sedation by one single, full trained endoscopist (A. G.). Prophylactic antibiotics were given for endocarditis prophylaxis when appropriate and in case of transrectal puncture. Staging of tumors was initially performed with a radial scanning echoendoscope (GF UM20; Olympus America Inc., Melville, NY). EUS-FNA was then carried out by using a curved linear array echoendoscope (GF UM30P; Olympus America Inc.) with Doppler capability and a scanning plane in the long axis of the instrument. A 22G needle-catheter system (Wilson-Cook Medical Inc., Winston-Salem, NC) was inserted through the working channel and advanced into the lesion under real-time ultrasound control, taking care not to pass through intervening vessels or the primary tumor (esophageal, gastric, and rectal cancer). After removal of the stylet, a 10-ml syringe was connected to the hub of the needle and a 3–4 ml suction applied as the needle was moved back and forth within the lesion. EUS-FNA was transesophageal, transgastric, or transrectal, depending on location of the lymph node. When more than one suspicious lymph node was observed in a particular patient, all of them were sampled. For each lymph node, the number of passes required to reach a final diagnosis and the order in which they were obtained were registered.

For the purpose of present study, results of conventional cytology and methylation analysis were compared with respect to the definitive diagnosis, which was established by either pathological examination of resected specimen or clinical follow-up in those cases not submitted to surgery. In this latter group, lesions were considered malignant in the case of clinical

metastatic progression of the disease or when an objective response was observed after chemotherapy or radiation therapy. On the contrary, lesions were considered benign when spontaneous resolution or lack of progression was observed on imaging studies after a minimum follow-up of 12 months. Cytological and molecular evaluations were performed in a blind fashion with respect to each other as well as to the definitive diagnosis.

Sensitivity, specificity, positive and negative predictive values, and overall accuracy of conventional cytology, methylation analysis, and combination of both techniques were calculated with respect to the definitive diagnosis in those patients in whom adequate material was obtained.

Conventional Cytology. The aspirated material was smeared onto glass slides and partially stained with a quick panoptic stain (Grifols, Barcelona, Spain) for immediate review by an on-site cytopathologist who verified adequacy of the specimen or advised as to the need for additional passes. The rest of aspirated material was preserved onto glass slides in ethanol for additional Papanicolaou staining and in saline for processing into a cellblock. Cytological material was considered adequate whether the attendant cytopathologist reported that there was a sufficient number of representative cells from the target lesion for the diagnosis or the presence of malignant cells was confirmed. In cytologically benign lymph nodes, the decision when to cease making needle passes was established taking into account meaningful clinical factors such as degree of clinical suspicious for underlying malignancy, clinical impact of a nondiagnostic aspirate, cytological appearance of the aspirated material, and the total number of passes (24).

Analysis of CpG Island Promoter Hypermethylation by Methylation-Specific PCR and Bisulfite Genomic Sequencing. Samples for methylation-specific PCR were obtained from aliquots of the saline rinse that was prepared for cellblock processing. They were collected and stored at -20°C for subsequent molecular analyses. DNA was extracted following standard procedures.

DNA methylation patterns in the CpG islands of *MGMT*, *p16^{INK4a}*, and *p14^{ARF}* were determined by chemical modification of unmethylated, but not the methylated, cytosines to uracil. Subsequently, PCR reactions were performed using primers specific for either methylated or the modified unmethylated DNA as described previously (25–27). One μg of DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega), treated with NaOH, precipitated with ethanol, and resuspended in water. Primer sequences and PCR conditions for the methylation analysis are available upon request. Placental DNA treated *in vitro* with *SssI* methyltransferase (New England Biolabs) was used as positive control for methylated alleles, and DNA from normal lymphocytes was used as negative control for methylated alleles. Controls without DNA were performed for each set of reactions. Ten μl of each PCR reaction was directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

Aberrant hypermethylation of the *MGMT*, *p16^{INK4a}*, and *p14^{ARF}* gene promoters were analyzed in gastrointestinal neoplasms, whereas methylation analysis was restricted to the

MGMT and *p16^{INK4a}* gene promoters in non-small cell lung cancer, according to the CpG island methylation profile of human tumors described previously (22). For the purpose of the current study, a lesion was considered positive when any of the evaluated gene promoters was hypermethylated.

All of the methylation-positive samples were verified to confirm their status by bisulfite genomic sequencing of the methylated-PCR product and by straight unbiased sequencing. For this last purpose, the following primers were used: p14^{ARF}-sense GGA GTT AGG AAT AAA ATA AGG GG, p14^{ARF}-antisense CCC CTT AAC TAC AAA CTA AAA CCC, p16^{INK4a}-sense TCCA ATT CCC CTA CAA ACT TC, p16^{INK4a}-antisense GAT TTG AGG GAT AGG GTA GGA GG, *MGMT*-sense GAG TTA GGT TTT GGT AGT GTT TAG G, and *MGMT*-antisense AAC CTA AAA CAA TCT ACR CAT CCC T. "R" represents either A or G.

Therapeutic Strategy. Treatment strategy was managed according to the therapeutic protocol of our center. The decision to perform preoperative chemotherapy and/or radiation therapy was driven by conventional cytology results, without considering those obtained in the methylation analysis.

Statistical Analysis. Continuous variables were expressed as mean \pm SD. Frequencies of conventional cytology, gene promoter hypermethylation, and their combination in benign and malignant lymph nodes were compared using the χ^2 test, applying the Yates correction when needed. A *P* value of 0.05 was considered statistically significant.

RESULTS

Twenty-seven patients (22 men; age, 66 \pm 9-years) were included in the study. In them, a total of 42 lymph nodes were sampled corresponding to patients with esophageal cancer (*n* = 11), rectal cancer (*n* = 7), gastric cancer (*n* = 3), or lung cancer (*n* = 21). According to the definitive diagnosis, 21 (50%) corresponded to metastatic lymph nodes (Table 1).

A total of 75 samples were obtained in these 42 lesions included in the study, thus representing 1.8 passes/lesion on average (range, 1–5 passes). At least 1 sample from each lesion was considered adequate for cytological diagnosis, whereas all 75 of the samples were useful for molecular analysis. Conventional cytology revealed neoplastic cells in 16 of 21 (76%) metastatic lymph nodes and in none of the benign lesions ($\chi^2 = 22.71$; *P* < 0.001). Aberrant methylation of gene promoters was detected in 17 of 21 (81%) metastatic lymph nodes, but they

were also detected in 7 of 21 (33%) benign lesions ($\chi^2 = 7.87$; *P* = 0.005). Presence of methylation at the CpG dinucleotides of the *p16^{INK4a}*, *p14^{ARF}*, and *MGMT* genes was also checked by bisulfite genomic sequencing of the methylated PCR products and by straight unbiased sequencing of the samples (Fig. 1). Taking into account these results, conventional cytology was considered more accurate than the methylation analysis for the diagnosis of metastatic lymph nodes (Table 2).

On the other hand, we were able to test 2 primary tumors of the 4 false-negative patients for methylation by both methylation-specific PCR and bisulfite genomic sequencing. These analyses showed that all 3 of the genes were unmethylated in the primary tumors, thus indicating that false-negative results were not due to technical limitations.

When both techniques were combined, 19 of 21 (90%) malignant lymph nodes and 14 of 21 (67%) benign lesions were correctly classified ($\chi^2 = 12.22$; *P* < 0,001). Combination of both techniques increased effectiveness of conventional cytology for the diagnosis of metastatic lymph nodes in terms of sensitivity and negative predictive value but decreased its specificity, positive predictive value, and overall accuracy (Table 2).

Hypermethylation of each specific gene promoter and particular type of cancer are depicted in Table 3. As it is shown, hypermethylation of the *MGTM* gene promoter had the highest sensitivity for the detection of occult malignant cells in any type of cancer.

DISCUSSION

The present study constitutes the first investigation demonstrating that it is feasible to detect lymph-node micrometastases by gene promoter hypermethylation analysis in aspirates obtained by EUS-FNA. This exploratory study also indicates that the molecular approach contribute to increase the sensitivity of conventional cytology in differentiating benign from malignant lymph nodes. These leading results probably reflect the combinatory effect of an endoscopic procedure with high proficiency for obtaining tissue samples from locoregional lymph nodes and a molecular analysis targeting tumor-associated DNA alterations involved in the own carcinogenic process.

Surgical resection remains the treatment of choice for patients with nonmetastatic gastrointestinal and lung tumors. In this setting, it is well known that long-term prognosis is determined by the pathological tumor stage, in which lymph node involvement is the main determinant. This fact not only has a prognostic significance but also it is critical for driving adjuvant therapies. In that sense, selection of an appropriate treatment strategy essentially depends on an accurate tumor staging. Recent studies have demonstrated that EUS-FNA, as a minimally invasive method of tissue sampling, is the most accurate technique for staging of esophageal, as well as a remarkable tool in gastric, rectal, and lung cancer (1–5). Among other factors, the accuracy of EUS-FNA depends on the pathologist capability in performing the cytomorphological analysis. On the other hand, even after extensive tumor staging, long-time survival remains unsatisfactory for most of these neoplasms, likely due to micrometastases undetectable by conventional diagnostic methods (28). Indeed, current histopathological techniques may fail to

Table 1 Primary tumor and definitive diagnosis of lymph nodes included in the study (*n* = 42)

Primary tumor	
Esophageal cancer	11 (26%)
Rectal cancer	7 (17%)
Gastric cancer	3 (7%)
Lung cancer	21 (50%)
Definitive diagnosis	
Benign lymph node	21 (50%)
Metastatic lymph node	21 (50%)
Criteria for definitive diagnosis	
Pathological examination of resected specimen	24 (57%)
Clinical follow-up	18 (43%)

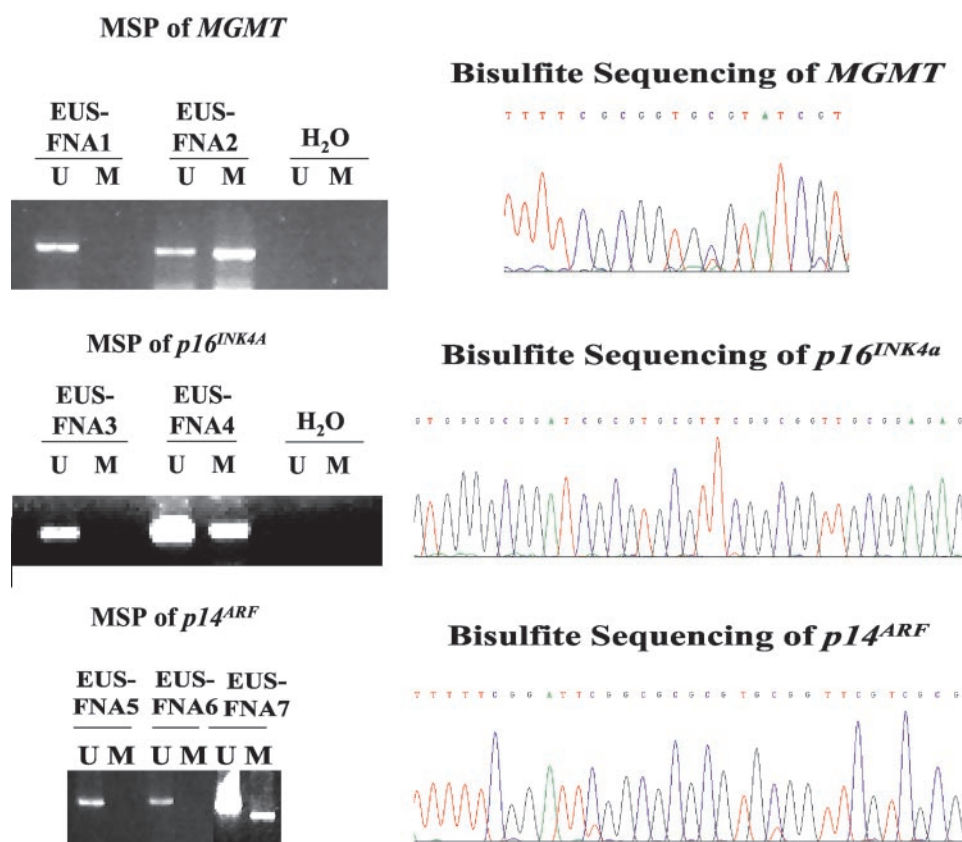


Fig. 1 Right, a representative example of the methylation-specific PCR analysis for *MGMT*, *p16^{INK4a}*, and *p14^{ARF}* gene promoters CpG islands. EUS-FNA2, EUS-FNA4, and EUS-FNA7 show hypermethylation of the *MGMT*, *p16^{INK4a}*, and *p14^{ARF}* gene promoters, respectively. Left, a representative example of the bisulfite genomic sequencing of those samples that were found methylated by methylation-specific PCR. The persistence of “Cs” preceding “Gs” after bisulfite modification indicates methylated cytosines.

detect lymph node metastases in sections having <2% neoplastic cells. Accordingly, both dependency on pathologist expertise and existence of undetectable micrometastases provided the rationale to combine the evaluation of cellular morphology and the analysis of tumor-associated DNA alterations.

To date, there are few data about the usefulness of EUS-FNA sampling for molecular analyses. Two studies have analyzed the utility of targeting *KRAS* gene for the diagnosis of pancreatic cancer in material obtained by EUS-FNA of pancreatic masses, because mutations of this gene are highly prevalent and specific in such a neoplasia. These investigations demonstrated that it was feasible to detect mutated *KRAS* in aspirates from the pancreas and that adding the molecular analysis im-

proved the efficacy of conventional cytology (29, 30). On the other hand, usefulness of EUS-FNA for the detection of micrometastases in lymph node has only been analyzed previously by Wallace *et al.* (31). In this study, it was possible to identify malignant lymph nodes in patients with non-small cell lung cancer targeting human telomerase reverse transcriptase mRNA. However, this strategy is limited by the necessity of handling RNA and the fact that telomerase activity has also been detected in several benign conditions characterized by lymphocyte infiltration, resulting in a high false-positive rate (57%; Ref. 14). To overcome these limitations, combination of targeting tumor-specific DNA mutations, which provide a high specificity, and the use of multiple markers, which would increase their sensi-

Table 2 Performance characteristics of conventional cytology, methylation analysis, and their combination, for the diagnosis of metastatic lymph node

Strategy	Sensitivity	Specificity	NPV ^a	PPV	OA
Conventional cytology	76% (16/21)	100% (21/21)	81% (21/26)	100% (16/16)	88% (37/42)
Methylation analysis	81% (17/21)	67% (14/21)	78% (14/18)	71% (17/24)	74% (31/42)
Combination of conventional cytology and methylation analysis	90% (19/21)	67% (14/21)	87% (14/16)	73% (19/26)	79% (33/42)

^a NPV, negative predictive value; PPV, positive predictive value; OA, overall accuracy.

Table 3 Performance characteristics of the methylation analysis for each specific gene promoter and particular type of cancer

Type of primary tumor	MGMT			<i>p16^{INK4a}</i>			<i>p14^{ARF}</i>		
	Sn ^a	Sp	OA	Sn	Sp	OA	Sn	Sp	OA
Esophageal cancer	67%	50%	64%	67%	50%	64%	33%	45%	45%
Gastric cancer	66%	-	66%	33%	-	33%	33%	33%	33%
Rectal cancer	80%	50%	71%	60%	50%	57%	60%	57%	57%
Any gastrointestinal cancer	71%	50%	67%	59%	50%	57%	41%	75%	48%
Lung cancer	50%	71%	67%	-	100%	81%	NA	NA	NA
Any gastrointestinal or lung cancer	67%	67%	67%	48%	90%	69%	NA	NA	NA

^a Sn, sensitivity; Sp, specificity; OA, overall accuracy; NA, nonavailable.

tivity, seems to be the most attractive approach to detect occult neoplastic cells.

In recent years, several investigations have demonstrated that methylation of CpG islands in promoter regions of several genes, including known tumor suppressor genes, results in the subsequent failure to express their functional proteins. Consequently, DNA methylation represents an early and fundamental step in the pathway by which normal tissue undergoes neoplastic transformation (17, 18). A unique profile of promoter hypermethylation exists for each human cancer in which some gene changes are shared and others are cancer-type specific (22). In that sense, gastrointestinal tumors share a set of genes undergoing hypermethylation including *p16^{INK4a}*, *p14^{ARF}*, *MGMT*, *APC*, and *hMLH1*, whereas lung cancer has a different pattern involving *DAPK*, *MGMT*, and *p16^{INK4a}* genes (22, 23). This spectrum of epigenetic alterations for a relatively small subset of genes constitutes a potentially powerful system of highly specific biomarkers. Taking into account all of these considerations, detection of neoplastic cell by aberrant gene promoter methylation appears to be an interesting molecular approach with potential clinical implications on diagnosis and prognosis.

In the present study, we demonstrated that it was feasible to perform the methylation analysis in all of the samples obtained by EUS-FNA from locoregional lymph nodes. The high performance of this approach is especially noteworthy considering the limited amount of material obtained in a single aspirate, which allowed for the analysis of at least three genes. Taking into account this limitation and based on data published previously, we considered that determination of gene promoter methylation pattern of *p16^{INK4a}*, *p14^{ARF}*, and *MGMT* genes for gastrointestinal cancer and of *p16^{INK4a}* and *MGMT* genes for lung cancer was the most proficient combination. We selected these markers because it has been demonstrated that methylation at these CpG sites is not present in normal tissue, it is associated with gene silencing, and, of note, these genes are methylated in a significant proportion of aerodigestive primary tumors (18, 22, 26). Similar results have also been shown in a recent study of detection of gene promoter hypermethylation in ductal lavage fluid and fine-needle washings from breast lesions (32, 33). In addition to the feasibility of this approach, results of present investigation indicated that it is possible to detect neoplastic cells in >80% of metastatic lymph nodes from esophageal, gastric, rectal, and lung cancer patients using a panel of only 2 or 3 genes. Moreover, as data from our analysis of correlation between aspirates and primary tumor samples showed, some false-negative results of the methylation analysis may actually

reflect the absence of markers in primary tumors, rather than a technical limitation. Overall, this high sensitivity accounted for the fact that adding the methylation analysis to conventional cytology resulted in the most sensitive strategy for detecting lymph node micrometastases. These results achieve a special relevance, because parallel studies have indicated that molecular detection of occult neoplastic cells in lymph nodes from patients with lung and colorectal cancer was a good predictor of tumor recurrence and survival (12, 15).

Limitations of the present study deserve some comments. First, sample size was limited, thus precluding drawing specific conclusions for each subtype of neoplasms. Nevertheless, results obtained in both gastrointestinal and lung cancer suggested that methylation gene promoter analysis might represent a potentially universal approach for the detection of micrometastases. Second, because some patients included in the study received preoperative or palliative chemoradiation therapy, definitive diagnosis based on pathological examination of resected specimen was not feasible in all of the patients. This fact, along with the lack of an unambiguous marker to be used as gold standard for occult lymph node micrometastases, may explain the relatively low specificity of the methylation analysis. Only longitudinal, follow-up studies will be able to elucidate the actual meaning of the detection of gene promoter hypermethylation in cytologically normal appearing lymph nodes.

In conclusion, results of the present study demonstrate that it is feasible to detect occult neoplastic cells in EUS-FNA samples by methylation gene promoter analysis. Moreover, addition of methylation analysis to conventional cytology may increase its sensitivity at the expenses of a decrease in its specificity. Additional investigations are needed to clarify the clinical relevance of molecular detection of micrometastases in patients with gastrointestinal and lung cancer.

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