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Association of *CK19 mRNA* detection of occult cancer cells in mediastinal lymph nodes in non-small cell lung carcinoma and high risk of early recurrence☆

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Abstract

This study was designed to screen occult cancer cells by *CK19 mRNA* detection using reverse transcriptase-polymerase chain reaction (RT-PCR) in mediastinal lymph nodes stations (MLNS) in non-small cell lung carcinoma (NSCLC). In 49 NSCLC patients free of mediastinal adenopathy on computed tomograph, 254 MLNS were evaluated by histopathology, immunohistochemistry (IHC) and RT-PCR. Of 225 non-tumoral MLNS on histopathology, 32 (14.2%) were positive by RT-PCR. IHC did not provide significant additional results. Seventeen patients were without mediastinal tumoral extension on histopathology and RT-PCR (Group 1), 16 were upgraded by RT-PCR (Group 2) and 16 pN2 on histopathology (Group 3). The two-year cancer-related death survival in Groups 1 (100%) and 2 (64.5%) was significantly different ($P = 0.04$). The relative risk of recurrence in Group 2 compared with Group 1, evaluated by the Cox model multivariate analysis, was 5.61 ($P = 0.02$). In conclusion, *CK19 mRNA* detected by RT-PCR in MLNS was significantly associated with an increased risk of rapid recurrence.
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Keywords: Cytokeratin; Reverse transcriptase polymerase chain reaction; Metastasis; Lung neoplasm; Thoracic surgery; Lymph node dissection; Mediastinum; Prognosis

1. Introduction

Curative surgery for lung cancer can be performed in only a few (25–30%) patients with non-small cell lung carcinoma (NSCLC). Current staging is still based on histological detection of malignant cells in regional lymph nodes, which remains the most accurate prognostic indicator and metastatic mediastinal lymph nodes are associated with a particularly poor prognosis [1,2]. Although stage I patients have been reported to have a better overall 5-year survival, 20–40% of them die from local recurrence or metastasis, mostly occurring during the two years following surgery [3]. Such data suggest that a proportion of pN0 patients are understaged by the usual staging procedures. In this context,

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detection of occult cancer cells either as micrometastases or isolated cancer cells is the subject of intense research using either immunohistochemistry (IHC) or molecular biology [4]. Occult lymph node micrometastases detected in this way have been demonstrated to be predictive of early recurrence or distant metastasis in a wide range of carcinomas [5]. In NSCLC, it has also been shown that occult micrometastases in mediastinal lymph nodes (MLN) detected by serial histological sections or IHC can be predictive of recurrence [6,7]. However, such a statement is still the subject of controversy [8]. As molecular methods, mostly based on reverse transcriptase-polymerase chain reaction (RT-PCR), have been shown to be more sensitive than IHC [2], we designed the present study to evaluate the use of RT-PCR, compared with IHC and histopathology, for the detection of occult tumour cells in MLN from a series of patients with NSCLC of the right lung undergoing surgery with systematic MLN dissection. Systematic MLN dissection has been shown to be an essential step in the intrathoracic staging of the disease without any increased morbidity and is a usual procedure in the care of our patients [9]. More recently, it has been clearly demonstrated that "examining a greater number of lymph nodes in patients with stage I NSCLC treated with resection increases the likelihood of proper staging and affects patient outcome" [10].

For RT-PCR, *cytokeratin (CK 19) mRNA* detection was chosen, as CK19 is expressed in NSCLC regardless of the subtype [11]. Although non-specific signals have been reported with *CK19 mRNA* RT-PCR [12], we, like other authors, have found that when well characterised procedures are used, pitfalls, such as detection of pseudogenes can be avoided [13,14].

2. Patients and methods

2.1. Patients

From December 1997 to November 1998, 57 patients with NSCLC affecting the right lung, who underwent curative-intent surgery were enrolled in the present study; 8 were excluded because of technical problems (*n*: 6) or histology (sarcoma: 1; carcinoid: 1). Patients who had received preoperative chemotherapy or who presented with metastasis or a previous malignant disease were not eligible. Forty nine patients were investigated further (Table 1). These included 10 women and 39 men, with a mean age of 62.4 years (range: 42–78 years), who were treated by pneumonectomy (35%, $n = 17$) or lobectomy (65%, $n = 32$) with MLN dissection as previously described in [15]. After surgery, 11 patients received mediastinal or chest wall radiotherapy for pT3 or pN2 tumours, and 5 patients received chemotherapy combined with radiotherapy. All patients gave their written consent and the study was approved by the Necker-Paris V Faculty of Medicine Ethics Committee.

3. Mediastinal lymph node examination

3.1. Lymph node dissection

The various MLN stations dissected were, according to Mountain and Dresler [16], stations 4R, 2R, and 7–11; station 4R (subdivided into 4 distinct levels), 2R and station 7 were evaluated by usual histopathology, IHC and RT-PCR. After careful dissection from the perinodal fat tissue, a series of 254 MLN stations [mean of 5.5 (range: 2–6) per patient] was studied (Table 1).

Each station was immediately divided into two parts: one for histopathology and IHC, the other snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction. The entire procedure from MNL dissection to freezing was performed within one hour.

3.2. Histology and immunohistochemistry

After acetic-formalin fixation for 24 h, specimens were cut into blocks 3 mm thick before embedding. Six serial 5 μm sections were taken from each block at approximately 50 μm intervals. The first and last sections were stained for histopathology; intermediate sections were incubated with anti-CK19 monoclonal antibody (Dako) (dilution: 1/100) and anti-pankeratin (Ventana 1:200). One section of each NSCLC was incubated with the anti-CK19 antibody as controls. Immunostaining was carried out as already described in [17] using the Ventana immunostainer automated method and the Ventana indirect biotin–avidin system DAB detection kit (Ventana Medical Systems).

3.3. CK19 mRNA detection by RT-PCR

Total RNA was extracted from the frozen specimens using the RNAzol method [19].

For *CK19 mRNA* RT-PCR, cDNA was reverse-transcribed from 2 μg of total RNA in a 20 μl reaction mix using the MMLV-Superscript II kit from Gibco-BRL (Life Technologies Inc.). CK19 nested PCR was performed as follows. Primers designed in order to avoid amplification of both genomic DNA and known processed pseudogenes were (A) 5′-AACCATGCAGAACCTCAACGACCGC-3′, (B) 5′-GGCAGGTCAGGAGAAGAGGC-3′ (1059 bp), (C) 5′-TCCCGCGACTACAGCC ACTACTACACGACC-3′, (D) 5′-CGCGACTTGATGTCCATGAGCCGCTGGTAC-3′ (745 bp). The upstream external primer was chosen to hybridise with exon 1 and the downstream primer with

Table 1
Patients characteristics according to nodal status divided into 3 groups

	Group 1[a]	Group 2[b]	Group 3[c]	Total
n	17	16	16	49
Mean age (range)	61.3 (45–78)	64.3 (42–74)	61.9 (49–74)	62.4 (42–78)
Gender				
Female	4	2	4	10
Male	13	14	12	39
Lobectomy	16	14	2	32
Pneumonectomy	1	2	14	17
Adenocarcinoma	9	6	11	26
SCC	8	10	5	23
T1	3	4	2	9
T2	12	10	8	30
T3	2	0	3	5
T4	0	2	3	5
2R + 4R + 7 MLNS (mean lymph nodes per patient)	5.5 (4–6)	5.5 (5–6)	5.4 (2–6)	5.5 (2–6)
Adjuvant therapy				
RT	0	0	11	11
CT	0	0	0	0
RT + CT	1	1	3	5

SCC, squamous cell carcinoma. 2R + 4R + 7 MLNS, mediastinal lymph node station both analysed in conventional histology, immunohistochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR).
RT, radiotherapy; CT, chemotherapy.
[a] Group 1: histopathology- and CK19 RT-PCR-negative.
[b] Group 2: histopathology-negative and CK19 RT-PCR-positive.
[c] Group 3: histopathology- and CK19 RT-PCR-positive.

exon 6. Both were chosen to contain mismatches with the pseudogene counterpart sequences. PCR was performed using 50 ng of cDNA with 18 pmol of primers in a reaction mix containing 50 mM Tris–HCl (pH 9), 50 mM KCl, 2 mM $MgCl_2$, 0.2 ng/ml bovine serum albumin (BSA), 16 mM $SO_4(NH_4)_2$, 500 μM deoxynucleotide triphosphates (dNTPs) and 0.2 U of Taq polymerase (ATGC) in a final volume of 25 μl. PCR was performed for 35 cycles at 94 °C for 50 s, 72 °C for 2 min 30 s, 72 °C for 1 min. 30 s, with a final extension at 72 °C for 10 min. In addition to mismatches with the pseudogene counterpart sequences, we verified that the PCR product contained a *Hae II* restriction site, missing within the pseudogene, in its 3′ terminal allowing the generation of two restriction fragments of 284 and 775 bp after *Hae II* digestion.

Internal PCR was performed on 1 μl of the final product of the first PCR reaction. Re-amplification was performed in a reaction mix containing 18 pmol of the internal primers. The sense primer was located in exon 1 and the antisense primer in exon 6. Except for $MgCl_2$ (4 mM), the reaction mix was similar to that used for the first PCR. This second round was performed for 35 cycles identical to the first round. As shown in Fig. 1, a positive result was registered when a 745 bp band was observed after gel electrophoresis.

To ensure completion of cDNA synthesis for each sample, β2-microglobulin (β2m) cDNA was amplified [18]. We carefully checked that no amplified product was obtained with 1 μg of genomic human DNA samples and cDNA obtained from RNA extracted from endothelial cells collected from scraped saphenous veins obtained from 3 patients who underwent venous surgery.

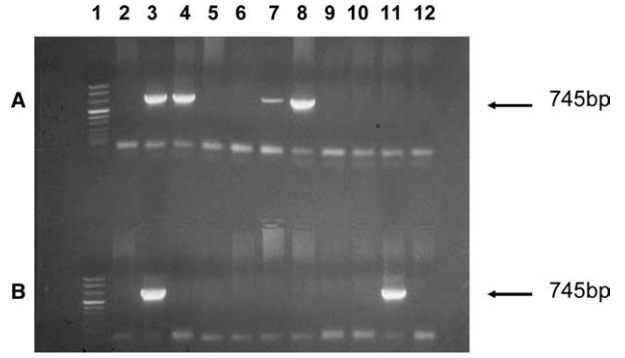

Fig. 1. Analysis of reverse transcriptase polymerase chain reaction (RT-PCR) amplification product (745bp) for detection of *CK19 mRNA* after polyacrylamide gel electrophoresis showing positive results for lymph nodes samples line A: lanes 3, 4, 7, 8 and line B: lanes 3 and 11.

3.4. Groups of patients, patient follow-up and statistical analysis

Groups of patients were defined according to the results obtained by, on the one hand, histopathology and/or IHC, and, on the other hand, *CK19mRNA*

detection by RT-PCR. The 3 groups were: Group 1 (histopathology- and CK19 RT-PCR-negative), Group 2 (histopathology-negative and CK19 RT-PCR-positive), group 3 (histopathology- and CK19 RT-PCR-positive).

Patients were examined every 3 months during the first year and then every year and earlier if any symptoms or signs of recurrence occurred. Recurrences were classified as local recurrences, contralateral tumours and distant metastases. Causes of death were coded as tumour-related or non-tumour-related. For all patients coded as alive, a cut-off date for the analysis of overall survival was taken at 4 years, with a median follow-up of 27 months.

The cumulative survival rates for patient groups were calculated by the Kaplan–Meier method and compared using the generalised Wilcoxon test. Recurrence-free survival taking into account local and distant recurrences was compared in the 3 groups. χ^2 tests and logistic regression were used to investigate the association between detection *CK19 mRNA* of RT-PCR and other prognostic variables. We assessed the effect of this detection on recurrence-free survival and overall survival controlling for the other prognostic factors with Cox's proportional hazards regression analysis. A P value of <0.05 was considered significant.

4. Results

4.1. Histopathology and immunohistochemistry

Carcinomatous cells were detected by histopathology in 29 mediastinal lymph node stations (MLNS) (Table 2). Compared with histopathology, additional results were obtained by IHC in 2 MLNS (Table 2) from patients with other MLNS metastatic by histopathology. These 31 MLNS were collected in 16 patients (32.6%) considered to be pN2 (Group 3) (Table 1). Histopathology was negative in the other 225 MLNS from 33 patients (Groups 1 and 2) (Table 1), 28 patients were classified as pN0 and 5 as pN1.

Table 2
Comparative results of histopathology, immunohistochemistry (IHC) and *CK19 mRNA* detection by RT-PCR

Number of lymph node stations	Histopathology	IHC	CK19 RT-PCR
23	+	+	+
6	+	+	–
2	–	+	+
30	–	–	+
193	–	–	–
Total: 254	29	31	55

4.2. CK19 mRNA detection by RT-PCR

As shown in Table 2, *CK19 mRNA* was detected by RT-PCR in 55 MLNS. After analysis of polyacrylamide gel electrophoresis (Fig. 1), the PCR product migrated at the expected size of 745 bp. *CK19 mRNA* was detected in 30 of the 223 samples (13.5%) from MLNS without carcinomatous cells detected by morphological methods. One hundred ninety three samples were negative whatever the methods used (4.2% of MLNS 2R, 13% of stations 4R and 8.2% of stations 7).

4.3. Restaging of N status according to CK19 RT-PCR

CK19 mRNA was detected by RT-PCR in 16 patients (48.5%) without metastatic MLNS according to histopathology. Three groups of patients (Table 1) were therefore defined: Group 1 (17 patients) without MLNS metastasis or occult tumour cells detected by RT-PCR, Group 2 (16 patients) with *CK19 mRNA* detected by RT-PCR without tumour cells observed by histopathology or IHC, and Group 3 (16 patients) with metastatic MLNS by histopathology (pN2).

5. Survival analysis

5.1. Overall and disease-free survival by univariate analysis

Overall survival at 2 and 4 years was 94.1%, 61.4%, 13.6% and 75.3%, 54.6%, 13.6% in Group 1, Group 2 and Group 3, respectively (Fig. 2(a)). The cancer-related death survival in the 3 groups is shown in Table 3 and in Fig. 2(b). At 2 years of follow-up, this survival was 100%, 64.5% and 22.5% in Groups 1, 2 and 3, respectively, with a significant difference between Groups 1 and 2 ($P = 0.04$). At 4 years, this survival was 80%, 58.2% and 22.5% (Table 3), with no significant difference between Groups 1 and 2.

At a follow-up of 2 years, the disease-free survival (Fig. 2(c)) was at the limit of the significance ($P = 0.056$) between Group 1 (87.5%) and Group 2 (59.2%). At 4 years, disease-free survival rates were 48.5%, 59.3% and 13.1% in Groups 1, 2 and 3, respectively.

5.2. Analysis of recurrences

At 4 years, 23 patients developed recurrence (Table 3). In Group 1, 5 patients developed metastases and 2 contralateral tumours (at 19 and 40 months). Six patients in Group 2 and 10 patients in Group 3 developed exclusively metastatic recurrence.

Time to recurrence differed between the three groups with a mean time of 31.5, 11 and 5 months in Groups 1, 2 and 3, respectively.

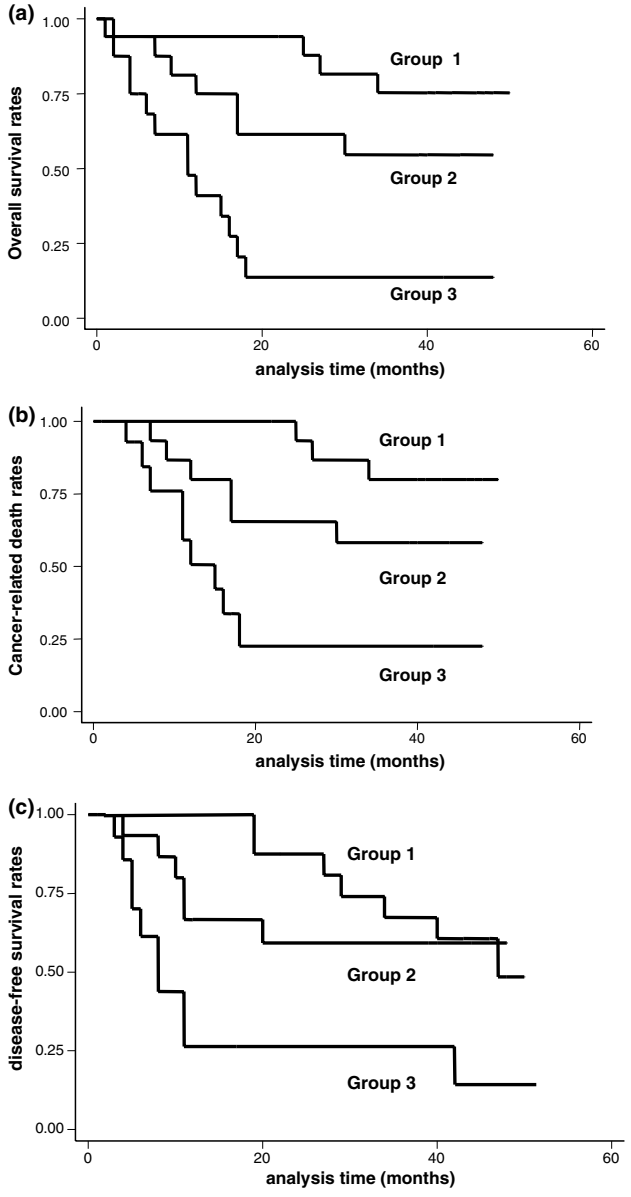

Fig. 2. Survival curves in the 3 groups of patients [Group 1: histopathology- and CK19 RT-PCR-negative; Group 2: histopathology-negative and CK19 RT-PCR-positive; Group 3: histopathology- and CK19 RT-PCR-positive (pN2)]: (a) overall survival curves; (b) cancer-related death curves; (c) disease-free survival curves.

5.3. Multivariate analysis

Patients in Group 2 had a significantly higher relative risk (RR) of earlier recurrence at 2-years compared with those in Group 1 ($P = 0.02$). Relative risks for Group 2 (RR = 5.6) and Group 3 (RR = 18.08) compared with Group 1 (Table 4), controlling for type of NSCLC, were significantly greater than 1 ($P = 0.0245$ and $P = 0.001$ for Groups 2 and 3, respectively). With a follow-up of 4 years, this significant difference was no longer observed ($P = 0.195$) for Group 2. The relative risk for squamous cell carcinoma (compared with adenocarcinoma) was not significantly different ($P = 0.07$).

6. Discussion

At the present time, staging of lymph node extension in NSCLC is exclusively based on histopathology. However, detection of occult tumour cells by more sensitive methods, i.e., IHC or molecular biology, remains a challenge, as it has been clearly demonstrated in various carcinomas that the presence of such cells has a significant impact on patient survival [4,19]. IHC has been used to detect occult tumour cells in lymph nodes or bone marrow in patients with NSCLC [6,7,19] and is considered to be a particularly sensitive method. However in the present study, it detected tumour cells in only 2 additional MLN considered to be negative by histopathology. Therefore, as already emphasised by Nicholson *et al.* [20] and recently by Marchevsky *et al.* [8], the present study did not emphasise the clinical value of IHC for evaluation of MLN in patients with NSCLC. Careful examination of serial sections by particularly experienced pathologists and the number of lymph nodes studed [10] can explain this absence of a significant difference between histopathology and IHC.

We tested RT-PCR based on *CK19 mRNA* identification for the detection of occult tumour cells. In previous published reports the use of *CK19 mRNA* as a marker of occult cancer cells has given very contrasting results. It has been shown to be particularly valuable for the detection of occult carcinomatous cells within lymph nodes in breast and cervical cancer [21]. In contrast, in other studies, including one in NSCLC, detection of *CK19 mRNA* by RT-PCR was considered to be non-specific [12]. Reported false-positive results were related to illegitimate ectopic expression, amplification of retrotransposon-like CK19 pseudogenes or epidermal cell contamination of blood samples [13,15,22–24]. However, in other series, when taking care to avoid these pitfalls, no false-positive results were reported either in lymph nodes [14] or other cells. In order to avoid non-specific amplification results, we, like others, designed our primers in divergent sequences between CK19 gene and pseudogenes [25] and we obtained no signal after amplification of genomic DNA or RNA from endothelial cells, as well as from 193 MLNS which is at variance with detection of *CK19mRNA* from lymph node non-epithelial cells. Indeed dendritic cells in lymph nodes which can be keratin-positive have been suspected to potentially give false-positive results. However, it has been shown that in contrast with the presence of cytokeratins 8 and 18, they do not contain CK19 [26].

RT-PCR detected *CK19 mRNA* in MLNS without tumour cells detected by histopathology or IHC and could therefore change the staging status in 16 patients

Table 3
Comparison of recurrences and cancer-related deaths in the 3 groups of patients

	Group 1[a] $n = 17$	Group 2[b] $n = 16$	Group 3[c] $n = 16$	Total $n = 49$
Number of recurrences	7	6	10	23
(% in each group)	(41.2%)	(37.5%)	(62.5%)	(46.9%)
Lost to follow-up[d]	1	1	1	3
Mean time to recurrence (months)	31.5	11	5	/
Deceased from recurrence	3	6	9	18
2-year survival (%)[e]	100[f]	64.5[f]	22.5	67.1
4-year survival (%)[e]	80	58.2	22.5	56.8

[a] Group 1: histopathology- and CK19 RT-PCR-negative.
[b] Group 2: histopathology-negative and CK19 RT-PCR-positive.
[c] Group 3: histopathology- and CK19 RT-PCR-positive.
[d] Without recurrence.
[e] Cancer-related death.
[f] The 2-year survival rates were significantly different between Groups 1 and 2 ($P = 0.04$).

Table 4
Predictive factors of prognosis at 2 and 4 years

Variable	Risk ratio at 2 years	P[a] value at 2 years	Risk ratio at 4 years	P value at 4 years
Group 2	5.61	0.0245	1.67	0.195
Group 3	18.08	0.001	6.58	0.001
Histology[b]	0.59	0.158	0.57	0.095

Relative risk of tumour recurrence was calculated by multivariate analysis with reference to Group 1.
[a] P value is the unilateral probability; i.e., 1/2 $P(>|z|)$.
[b] Comparing squamous cell carcinomas with adenocarcinomas.

(48.5%). Similar results demonstrating the efficiency of molecular markers to detect occult micrometastases have already been reported [2,27,28] as in breast cancer [5], where RT-PCR was found to be more sensitive and less expensive than serial-sectioning and IHC staining. One reservation concerns the false-negative results obtained by RT-PCR previously reported in [24,29], which can be hypothesised to result from the use of only one molecular marker with heterogeneous expression in tumour cells [30] or the sampling of nodes with rare and occult tumour cells localised in a restricted area. The known uneven distribution of occult metastases within lymph nodes could be one explanation of the results which differed between histopathology and CK19 RT-PCR.

The correlation between occult cancer cells in MLNS detected by molecular biology and prognosis is still a subject of controversy [27]. In the present study, the survival analysis and the pattern of tumour recurrence in patients upgraded by *CK19 mRNA* RT-PCR were clearly different to those observed in patients found to be negative by histopathology and RT-PCR, particularly during the first 2 years of follow-up. After 4 years, the difference was no longer significant for disease-free survival, which could be explained by the small patient population evaluated in each group. The pattern of recurrence was also different: patients negative by histopathology and RT-PCR developed contralateral tumours or metastases, while patients upgraded by *CK19mRNA* RT-PCR and pN2 patients exclusively developed metastatic disease. The time to recurrence was also clearly shorter in patients upgraded by *CK19 mRNA* RT-PCR.

This clinical difference between patients with CK19 RT-PCR-negative or CK19 RT-PCR-positive lymph nodes provides an additional convincing argument that *CK19 mRNA* detection can be considered to be a marker of occult tumour dissemination, indicating a high risk of early diffusion of the disease, in this study and in other studies [4,19].

In conclusion, the significance of occult tumour cells in MLNS remains unclear, but this study shows that the presence of these cells is associated with a poorer prognosis and earlier recurrence. This preliminary study should encourage us to look in a larger series of patients for RT-PCR analysis of mediastinal lymph nodes in order to validate the use of CK19 RT-PCR detection in the routine staging assessment of patient with NSCLC. Therefore, in order to identify patients at an increased risk for early recurrence. The objective of this screening would be to identify a group of patients with a high risk of recurrence after surgery, who would be eligible for adjuvant therapy. The clinical implication of CK19 RT-PCR therefore needs to be evaluated in a larger number of patients in order to confirm these preliminary results.

Conflict of interest statement

None declared.

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