PERGAMON

European Journal of Cancer 38 (2002) 2325–2330

European Journal of Cancer

www.ejconline.com

Detection of circulating cells expressing *chromogranin A* gene transcripts in patients with lung neuroendocrine carcinoma

H. Bégueret[a,*], B. Vergier[a], J. Bégueret[b], J.M. Vernejoux[c], J. Jougon[d], R. Trouette[e], A. Taytard[c], J.F. Velly[d], J.P. Merlio[a], A. de Mascarel[a]

[a]*Service d'Anatomie Pathologique, Hôpital Haut-Lévêque, Avenue Magellan, 33604 Pessac Cedex, France*
[b]*Laboratoire de Génétique Moléculaire, Institut de Biochimie et Génétique Cellulaires, UPR CNRS 9026, France*
[c]*Service de Pneumologie, Hôpital Haut-Lévêque, CHU Bordeaux, France*
[d]*Service de Chirurgie Thoracique, Hôpital Haut-Lévêque, CHU Bordeaux, France*
[e]*Service de Radiothérapie, Hôpital Saint-André, CHU Bordeaux, France*

Received 21 January 2002; received in revised form 7 May 2002; accepted 13 August 2002

Abstract

High grade lung neuroendocrine carcinomas, like small and large cell neuroendocrine carcinomas, pose therapeutic problems. Most initially respond to chemotherapeutic agents, but early relapses are frequent and are resistant to the presently available treatments. Our study reports for the first time the development and evaluation of a test for detecting the presence of circulating tumour cells by measuring *chromogranin A* gene transcripts with reverse transcriptase-polymerase chain reaction (RT-PCR) and Southern blotting. The test is specific and sensitive (detection of 10 cancer cells/ml blood), and only minimally invasive. Positivity is statistically correlated to high grade neuroendocrine carcinomas and to a poor prognosis with a 3-fold higher lethal risk. The test now needs to be assessed for its usefulness as a tool in the initial staging procedures and follow-up by comparison with the recent immunoradiometric assay (RIA) for detection of chromogranin A in the serum.

Keywords: Circulating cells; *Chromogranin A* transcripts; Neuroendocrine carcinoma

1. Introduction

The concrete applications of an ideal biological marker are an early diagnosis, discrimination between benign and malignant neoplasms, follow-up of the disease and therapeutic efficiency, and appreciation of the tumour burden. Two classes of tumour markers are now available: serum protein markers and detectors of circulating cancerous blood cells. The latter has now been widely studied in various neoplasms such as breast carcinoma [1], prostatic adenocarcinoma [2], colorectal adenocarcinoma [3,4], melanoma [5], neuroblastoma [6], and non-neuroendocrine lung carcinomas [7,8] but never in neuroendocrine tumours such as pulmonary neuroendocrine carcinomas.

Chromogranin A (CgA) is a 48-kDa protein whose immunohistochemical detection is a very useful tool in the diagnosis of neuroendocrine tumours. This member of the granin protein family is distributed in secretory dense core granules of neuroendocrine cells and is structurally characterised by numerous mono- and dibasic sites. Both inside and outside these granules, CgA undergoes proteolytic cleavage and is released as bioactive peptides with tissue specificity such as pancreastatin, vasostatin/β-granin, chromostatin, WE-14, parastatin/GE-25 and prochromacin [9–12].

Such a complex tissue-specific proteolysis may explain the difficulties experienced in developing sensitive and specific assays for the detection of circulating CgA protein. CgA is now gaining acceptance as a serum marker of neuroendocrine tumours since the recent report of a new human CgA immunoradiometric assay (RIA) involving monoclonal antibodies raised against the unprocessed central domain [13].

* Corresponding author. Tel.: +33-5-56-55-64-75; fax: +33-5-56-55-23-72.
E-mail address: hugues.begueret@chu-bordeaux.fr (H. Bégueret).

0959-8049/02/$ - see front matter
PII: S0959-8049(02)00453-7

Reverse transcriptase-polymerase chain reaction (RT-PCR) assays have been used for the detection of several classes of circulating tumour cells in the blood and bone marrow. We have used it as an alternative to serum CgA detection and now report for the first time its use to detect circulating tumour cells expressing neuroendocrine markers in patients with lung neuroendocrine carcinoma. Other aims of this study were to evaluate the clinical usefulness of this new neuroendocrine blood marker in surveillance of the clinical and therapeutic response.

2. Materials and methods

2.1. Patients

Thirty-six unselected patients with newly diagnosed neuroendocrine lung carcinoma (25 males, 11 women; mean age 64.3 years; range 44–85 years) referred to the university hospital of Bordeaux were enrolled in this study. Diagnosis included typical carcinoid (TC) ($n=8$), neuroendocrine large cell carcinoma (LCC) ($n=5$) and small cell carcinoma (SCLC) ($n=23$). No atypical carcinoid was included. Twenty-one patients (60%) had limited disease (7 TC, 4 LCC and 10 SCLC), and 14 (40%) had extensive disease with distant metastasis (1 TC, 1 LCC and 12 SCLC).

2.2. Tumour samples

Fresh tumour specimens from non-neuroendocrine and neuroendocrine lung carcinomas were snap frozen in liquid nitrogen and stored at −80 °C before RNA extraction. Formalin-fixed and paraffin-embedded tumour specimens from each patient were reinterpreted to obtain a diagnosis according to the World Health Organization (WHO) and Travis classifications of neuroendocrine lung carcinomas [14]. In all the cases, an immunohistochemical study with NSE (DAKO, Netherlands), CgA (Biogenex, France) and Ki67 (MiB1) (DAKO, Netherlands) was performed.

2.3. Blood samples

Five millilitres of blood were collected in ethylene diamine tetra acetic acid (EDTA) tubes from each patient before treatment of their neuroendocrine lung carcinoma. Twenty-three patients were selected as controls, two with a non-neuroendocrine lung carcinoma, one with a B-cell lymphoma and twenty healthy volunteers.

2.4. Cell lines

Four cell lines of neoplastic cells from SCLC were gratefully provided by Dr H.K. Oie from the National Cancer Institute-National Naval Center (Bethesda, Maryland). The H419 cell line was used for reconstitution experiments, sensitivity and specificity testing. Ten to 10^7 H419 cells were diluted in blood samples from healthy control subjects. Cells were subsequently isolated by Ficoll-Hypaque density centrifugation.

2.5. RNA purification

Total cellular RNA was extracted using the "High Pure RNA Isolation Kit" (Boehringer Mannheim). Quantification and evaluation of the RNA integrity of each preparation were performed by absorbance at 260 nm and *β-actin* RT-PCR.

2.6. Oligonucleotide primers and probes

Oligonucleotide primers and probe sequences for *β-actin*, *γNSE* (nueron specific enolase) and *chromogranin A* were selected from the Genebank international data bank and with the "Oligo" program. To avoid spurious amplification of genomic DNA, each primer set was selected from two different exons with at least one intervening intron.

Human *β-actin* primers were as follows: primer I: 5′-CTACAATGAGCTGCGTGTGGC-3′ and primer II: 3′-CACAGTGTGGGTGACCCCGT-5′. *γNSE* primers and probe sequences were as follows: primer I (on exon 8): 5′-TCTCCCACTGATCCTTCCCG-3′; primer II (on exon 9): 3′-TCCACTGCCCGCTCAATACG-5′; and probe: 5′-CTATCCTGTGGTCTCC-3′. *Chromogranin A* primers and probe were as follows: primer I (on exon 7): 5′-GAGTGGGAGGACTCCAAACG-3′; primer II (on exon 8): 3′-CCACTTTCTCCAGCTCTGCC-5′; and probe: 5′-CCGCAGACCAGAGGACCAGGAGCTGG-3′.

2.7. Reverse transcription and polymerase chain reaction (PCR) amplification

Reverse transcription and PCR were performed in one tube using the "Access RT-PCR System" (Promega) in a 50 μl volume. The reaction was performed in a thermocycler under the following conditions: 45 min of reverse transcription at 48 °C and 2 min of AMV reverse transcriptase inactivation, and 40 amplification cycles: denaturation (1 min; 95 °C), hybridisation (1 min; 60 °C), and annealing (1 min; 72 °C).

Control reactions were always performed using the same reagents as for the experimental samples, but without RNA.

2.8. Gel electrophoresis and Southern-blotting

After completion of the amplification cycles, 10 μl of each PCR product were run in a 1.5% agarose gel containing ethidium bromide. PCR products were transferred

onto a positively charged nylon membrane (Hybond-N; Amersham) by overnight alkali capillary blotting with the use of 0.25 M NaOH/1.5 M NaCl. DNA probes were labelled with γ^{32}P adenosine triphosphate (ATP) (Amersham) using T4 polynucleotide kinase (Promega). Prehybridisation was performed in a solution containing 6× standard sodium citrate (SSC), EDTA 5 mM, sodium dodecyl sulphate (SDS) 0.1% and skimmed milk 2.5% (w/v) at 65 °C for 1 h. After this time, the labelled probe was added to the solution, and hybridisation was allowed to proceed overnight. Filters were washed twice in 2× SSC, 0.1% SDS at 65 °C for 15 min and then in 0.5 SSC, 0.1% SDS at 65 °C for 15 min. Hybridisations were revealed with a Phosphoimager.

2.9. Immunohistochemical study

Immunohistochemical studies were performed on 3-μm paraffin-embedded sections in the Techmate 500 DAKO with "Universal DAKO LSAB kit, peroxidase" (DAKO, Netherlands) with commercial antibodies against γNSE, chromogranin A and Ki67 (MiB1).

2.10. Statistics

Statistical analysis was performed with a Chi2 test according to the Miettinen method.

3. Results

3.1. Evaluation of RT-PCR specificity in detecting γNSE and chromogranin A transcripts

RNA integrity and quantification were performed in all samples by the detection of a 218-bp *β-actin* product in ethidium bromide-stained gels. Ethidium bromide staining of the amplified *γNSE* and *chromogranin A*

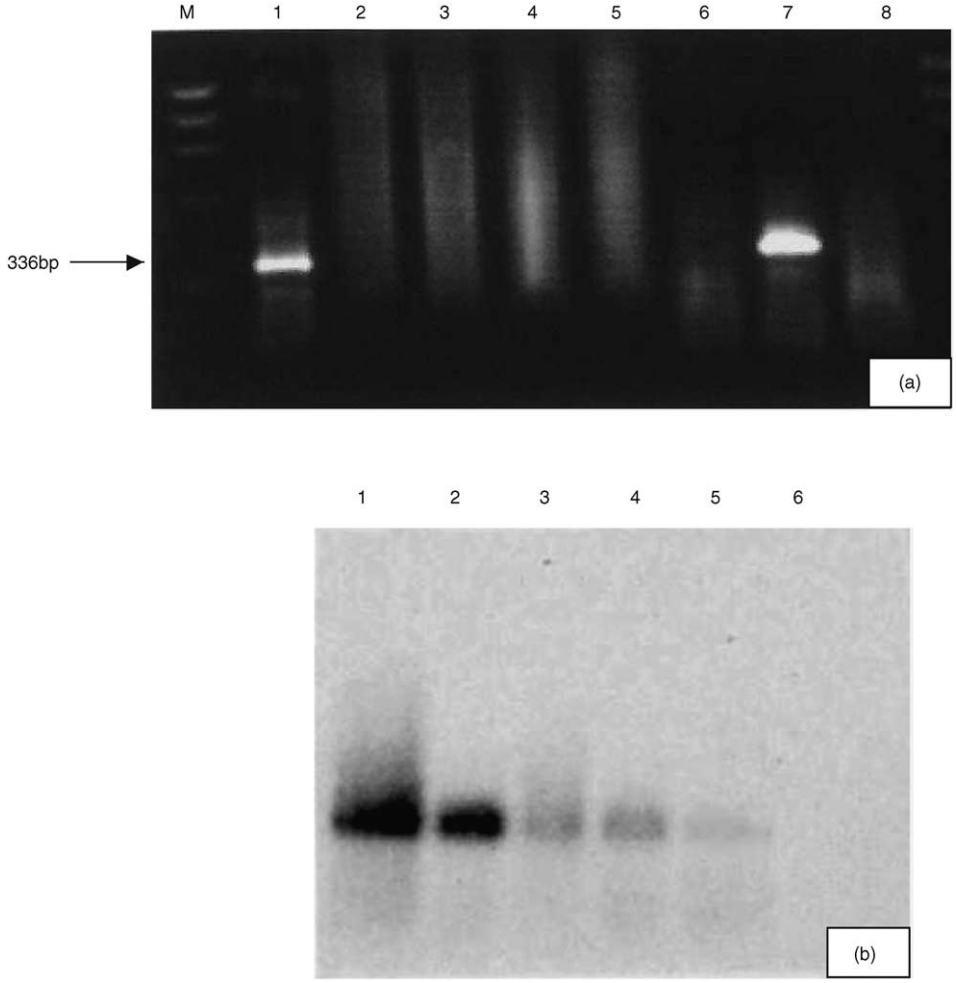

Fig. 1. Evaluation of reverse transcriptase-polymerase chain reaction (RT-PCR) sensitivity in detecting *chromogranin A* transcripts by performing serial dilutions of H419 cells in 1 ml of whole blood from healthy volunteers. M: molecular weight marker (ΦX174 DNA restricted by *Hae III*), 1: 10^5 cells/ml blood, 2: 10^4 cells/ml blood, 3: 10^3 cells/ml blood, 4: 10^2 cells/ml blood, 5: 10 cells/ml blood, 6: healthy volunteer blood control, 7: positive control, 8: negative control (water). 1a—Agarose gel electrophoresis of RT-PCR products. 1b—Hybridisation of RT-PCR products with a specific internal radiolabelled oligonucleotidic probe.

gene products from total RNA of H419 tumour cells revealed bands of the expected molecular size (214 bp for *γNSE* and 336 bp for *chromogranin A*). Hybridisation of the PCR products with specific radiolabelled internal oligonucleotidic probes confirmed the specificity of the RT-PCR.

Blood specimens from healthy volunteers and tumour samples from patients with lung adenocarcinoma, lung squamous carcinoma and lymphoma were examined using RT-PCR and Southern blotting. Each of them contained detectable *γNSE* transcripts. In contrast, they did not contain detectable *chromogranin A* transcripts. Therefore, chromogranin A was retained as a specific marker for the detection of tumour cells.

3.2. *Evaluation of RT-PCR sensitivity in detecting* chromogranin A *transcripts*

Sensitivity of the detection of *chromogranin A* transcripts was determined by performing serial dilutions of H419 cells in 1 ml of whole blood from healthy volunteers to represent 10^5, 10^4, 10^3, 10^2 and 10 tumour cells/ml blood. Each sample mix was tested by RT-PCR and Southern blotting. mRNA of *chromogranin A* could be detected by ethidium bromide staining only in the sample containing 10^5 tumour cells/ml blood (Fig. 1a). By Southern blotting, *chromogranin A* mRNA was detected at a concentration as low as 10 tumour cells/ml whole blood (Fig. 1b).

3.3. *Detection of* chromogranin A *mRNA expression in the blood of 36 patients with lung neuroendocrine carcinomas*

mRNA (250 ng) from each sample were analysed by RT-PCR and Southern blotting with specific primers and a probe to detect *chromogranin A* transcripts. The evaluation of the amplified signal after hybridisation was semi-quantitative (Fig. 2).

Blood specimens from healthy subjects and from patients with lung adenocarcinoma, lung squamous carcinoma and lymphoma were examined as controls. In accordance with the preceding results, all of these controls were negative after RT-PCR and Southern blotting (Table 1).

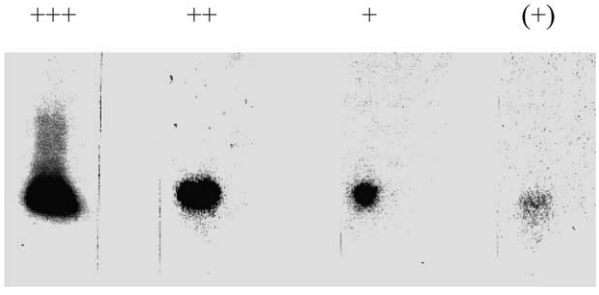

Fig. 2. Examples of semi-quantitative evaluation of the amplified signal after hybridisation: (+): approximately 10–10^2 cancer cells/ml blood; +: approximately 10^2–10^4 cancer cells/ml blood, ++: approximately 10^4–10^5 cancer cells/ml blood, +++: about $>10^5$ cancer cells/ml blood.

3.4. *Results assessed according to histological type of neuroendocrine tumour*

No *chromogranin A* transcripts were detected for patients with typical carcinoid even for the patient with hepatic metastasis. In contrast, 52% of the SCLC cases and 80% of the LCC cases gave a positive signal of varying intensity, thus indicating a statistical significance between the degree of malignancy of the neuroendocrine carcinoma and the positivity of the test ($P=0.017$) (Table 1).

3.5. *Results according to the patients' clinical status at the time of blood sampling and their evolution (Table 2)*

64% of patients with extensive disease were positive in contrast to 33% with localised disease. Such a difference did not achieve statistical significance ($P=0.07$). However, among the positive patients (33%) with localised disease, 57% had developed metastases. Localisations were liver, brain, bone, adrenal gland and bone marrow.

When the analysis was restricted to high grade neuroendocrine carcinomas (SCLC and LCC), there was a statistical significance ($P<0.05$) between the positivity of the test and the evolution of the patients. In fact, 81.2% of the positive patients presented with metastases either at the time of diagnosis or thereafter.

3.6. *Survival analysis*

62.5% of the patients with a positive test died with a 6.8 months mean survival (range 0.5–20 months). This is in contrast to 26.3% of the negative patients (5 months mean survival; range 1–11 months). Therefore, there was a statistically significant difference between test positivity and outcome ($P=0.03$). The risk of death when the test was positive was approximately three

Table 1
RT-PCR analysis of blood specimens from the tumour-bearing patients and controls

Tumour status	Number of patients	RT-PCR results	
		Negative	Positive
SCLC	23	11	12
LLC	5	1	4
Typical carcinoid	8	8	0
Non neuroendocrine lung tumor	3	3	0
Normal controls	20	20	0

LCC, neuroendocrine lung large cell carcinoma; SCLC, small cell lung carcinoma. RT-PCR, reverse transcriptase-polymerase chain reaction.

times higher (95% Confidence Interval (CI) = 1.09–8.16).

4. Discussion

Due to their neuroectodermal origin, neuroendocrine carcinomatous cells produce a wide variety of molecules that are potentially usable as tumour markers such as enzymes, secretory peptides, hormones and surface molecules. Most have been tested as serum protein markers, but none have proved their superiority. Reverse transcriptase and in vitro amplification (RT-PCR) is the most sensitive method for the detection of isolated tumour cells either in blood samples or in tissue specimens. Such tests used to detect specific mRNA from tumour cells can provide a better staging of disease by the detection of circulating blood cells or lymph node micrometastases.

Most of the reported RT-PCR tests have a similar sensitivity of one tumour cell per 10^7 mononuclear blood cells. In contrast, various studies have shown wide variations for the specificity of positive results 8,15]. One hypothesis for the difference of specificity is the accidental amplification of pseudogenes [15].

The present paper presents for the first time a test for detecting circulating blood cells expressing *chromogranin A* messengers. As regards *γNSE* mRNA, their abundance in blood cells precludes their potential use as a marker.

The test is specific and sensitive allowing the detection of 10 cancer cells/ml blood. The negativity of the assay refers to samples with less than 10 cancer cells/ml blood. Its application to a 36-patient cohort with neuroendocrine

Table 2
Patients' clinical characteristics and RT-PCR results

Case	Sex	Age (years)	Diagnostic	Extension	RT-PCR[a]	Follow-up (months)
1	F	46	TC	L	–	27
2	F	48	TC	L	–	53
3	M	49	TC	E (liver)	–	8
4	F	61	TC	L	–	9
5	F	75	TC	L	–	38
6	F	74	TC	L	–	10
7	M	73	TC	L	–	12
8	F	44	TC	L	–	41
9	M	61	LCC	E (liver, adrenal gland)	++	0.5
10	M	48	LCC	L	+	20
11	M	66	LCC	L	+	17
12	M	60	LCC	L	(+)	18
13	M	71	LCC	L	–	1
14	F	82	SCLC	L	(+)	2
15	M	59	SCLC	E (brain)	+	10
16	M	71	SCLC	E (bone, liver)	+++	2
17	M	51	SCLC	E (pancreas, liver)	+	13
18	M	48	SCLC	L	+	11
19	M	66	SCLC	E (brain)	++	13
20	M	73	SCLC	L	++	2
21	M	64	SCLC	E (bone marrow)	+	8
22	F	76	SCLC	E (bone)	+	7
23	F	77	SCLC	L	–	11
24	M	75	SCLC	L	–	6
25	M	54	SCLC	L	–	8
26	F	57	SCLC	L	+	11
27	M	73	SCLC	E (brain, liver)	–	7
28	M	66	SCLC	E (liver)	–	9
29	M	60	SCLC	E (brain, adrenal gland)	+	13
30	M	60	SCLC	L	–	2
31	M	52	SCLC	E (liver, bone)	+++	5
32	M	70	SCLC	L	–	11
33	F	53	SCLC	E (liver, bone, adrenal gland)	–	7
34	M	84	SCLC	E (liver)	–	LFU
35	M	85	SCLC	E (liver, bone)	–	5
36	M	84	SCLC	L	–	12

E, extensive disease; F, female; L, limited disease; LFU, lost to follow-up; LCC, neuroendocrine lung large cell carcinoma; M, male; SCLC, small cell lung carcinoma; TC, typical carcinoid.

[a] Semi-quantitative evaluation of the amplified signal after hybridisation. (+): approximately 10–10^2 cancer cells/ml blood; +: approximately 10^2–10^4 cancer cells/ml blood; ++: approximately 10^4–10^5 cancer cells/ml blood; +++: approximately $>10^5$ cancer cells/ml blood (Fig. 2).

lung carcinoma shows its prognostic value with a risk of death three-fold higher for the positive patients. Moreover, the test when restricted to high grade neuroendocrine carcinomas (SCLC and LCC) correlated well with the clinical staging. Current clinical staging procedures may not detect these microscopical carcinomatous disseminations, so patients may be considered as negative. However, we show here that such disseminations may now be detected due to the sensitivity of this test. Moreover, the test's initial interpretation as being a false-positive was not in fact confirmed, since many of these patients did develop metastases.

It has been proposed that not all circulating cancer cells develop metastases. Such an observation was made in recent studies following the detection of positive prostate-specific antigen (PSA) circulating cells and circulating human *lipase* mRNA in patients with hepatocellular carcinoma [16,17]. On the other hand, false-negatives could be explained by an intermittent blood dissemination of cancer cells due to chronobiological factors.

In conclusion, our study reports for the first time the development and evaluation of a test for detecting the presence of circulating tumour cells by measuring *chromogranin A* gene transcripts with RT-PCR and Southern blotting in patients with lung neuroendocrine carcinoma. This specific and sensitive test is statistically correlated to high grade neuroendocrine carcinomas and to those patients with a poor prognosis (3-fold higher lethal risk). It would now be advisable to apply this new test to a larger cohort of patients in order to clarify its usefulness in staging and in monitoring lung neuroendocrine carcinoma patients compared with the efficacy of the recently reported RIA used for the detection of human chromogranin A in the serum [13].

Acknowledgements

This work was supported by the Comité des Charentes de la Ligue Contre le Cancer. We thank Dr Laurent Portel and Dr Laurent Falque for their clinical assistance and Dr Xavier Jacquelin for statistical analysis. We are indebted to Martine Sabourin, Jackie Ferrer and J.C. Garoste for technical assistance.

References

1. Leitzel K, Lieu B, Curley E, *et al*. Detection of cancer cells in peripheral blood of breast cancer patients using reverse transcription-polymerase chain reaction for epidermal growth factor receptor. *Clin Cancer Res* 1998, **4**, 3037–3043.
2. Seiden MV, Kantoff PW, Krithivas K, *et al*. Detection of circulating tumor cells in men with localized prostate cancer. *J Clin Oncol* 1994, **12**, 2634–2639.
3. Burchill SA, Bradburry MF, Pittman K, Southgate J, Smith B, Selby P. Detection of epithelial cancer cells in peripheral blood by reverse transcriptase-polymerase chain reaction. *Br J Cancer* 1995, **71**, 278–280.
4. Wyld DK, Selby P, Perren TJ, *et al*. Detection of colorectal cancer cells in peripheral blood by reverse-transcriptase polymerase chain reaction for cytokeratin 20. *Int J Cancer* 1998, **19**, 288–293.
5. Smith B, Selby P, Southgate J, Pittman K, Bradley C, Blair GE. Detection of melanoma cells in peripheral blood by means of reverse transcriptase and polymerase chain reaction. *Lancet* 1991, **338**, 1227–1229.
6. Mattano LA, Moss TJ, Eerson SG. Sensitive detection of rare circulating neuroblastoma cells by the reverse transcriptase-polymerase chain reaction. *Cancer Res* 1992, **52**, 4701–4705.
7. Dingemans AM, Brakenhoff RH, Postmus PE, Giaccone G. Detection of cytokeratin of cytokeratin-19 transcripts by reverse transcriptase-polymerase chain reaction in lung cancer cell lines and blood of lung cancer patients. *Lab Invest* 1997, **77**, 213–220.
8. Krismann M, Todt B, Schroder J, *et al*. Low specificity of cytokeratin 19 reverse transcriptase-polymerase chain reaction analyses for detection of hematogeneous lung cancer dissemination. *J Clin Oncol* 1995, **13**, 2769–2775.
9. Barbosa JA, Gill BM, Takiyyuddin MA, Connor DT. Chromogranin A: post-translational modifications in secretory granules. *Endocrinology* 1991, **128**, 174–190.
10. Metz-Boutigue MH, Garcia-Sablone P, Hogue-Angeletti R, Aunis D. Intracellular and extracellular processing of chromogranin A. Determination of cleavage sites. *Eur J Biochem* 1993, **217**, 247–257.
11. Iacangelo AL, Eiden LE. Chromogranin A: current status as a precursor for bioactive peptides and a granulogenic/sorting factor in the regulated secretory pathway. *Regul Pept* 1995, **58**, 65–88.
12. Strub JM, Goumon Y, Lugardon K, *et al*. Antibacterial activity of glycosylated and phosphorylated chromogranin A-derived peptide 173–194 from bovine adrenal medullary chromaffin granules. *J Biol Chem* 1996, **271**, 28533–28540.
13. Degorce F, Goumon Y, Jacquemart L, *et al*. A new human chromogranin A (CgA) immunoradiometric assay involving monoclonal antibodies raised against the unprocessed central domain (145–245). *Br J Cancer* 1999, **79**, 65–71.
14. Travis WD, Colby TV, Corrin B, Shimosato Y, Brambilla E. World Health Organization. *Histological Typing of Lung Tumors*. 3rd edn. Springer, 1999.
15. Zippelius A, Kufer P, Honold G, *et al*. Limitations of reverse-transcriptase polymerase chain reaction analyses for detection of micrometastatic epithelial cancer cells in bone marrow. *J Clin Oncol* 1997, **15**, 2701–2708.
16. Thiounn N, Saporta F, Flam TA, *et al*. Positive prostate-specific antigen circulating cells detected by reverse transcriptase-polymerase chain reaction does not imply the presence of prostatic micrometastases. *Urology* 1997, **50**, 245–250.
17. Leonhardt U, Ritzel U, Ottleben M, Ramadori G. Circulating human hepatic lipase mRNA in patients with hepatocellular carcinoma and healthy controls. *Eur J Surg* 1999, **165**, 539–542.