Full Length Research Paper

Detection of circulating tumor cells in the peripheral blood of nasopharyngeal carcinoma patients by a nested reverse transcriptase polymerase assay for cytokeratin 19 mRNA

S. AISSI-BEN MOUSSA[1,2], F. GUEMIRA[3] and M. P. BUISINE[2,4,5]

[1]Laboratoire de Biochimie et Biologie Moléculaire, Faculté des Sciences de Tunis, Tunis, Tunisie.
[2]INSERM U837, Centre de Recherche JP Aubert, Equipe n°5, Lille, France.
[3]Service de Biochimie clinique, Institut Salah AZAIEZ de Tunis, Tunis, Tunisie.
[4]Laboratoire de Biochimie et Biologie Moléculaire, CHRU de Lille, Lille, France.
[5]Faculté de Médecine H. Warembourg, Université Lille 2, Lille, France.

Nasopharyngeal carcinomas (NPC) are tumors of nasopharynx origin with high rate of distance metastases after radiotherapy. Therefore, detection of micrometastasis will be an important issue in the prognostic and the choice of the systemic treatment. Our aim was early detection of circulating tumor cells in the blood of NPC patients by an RT-PCR assay for cytokeratin19 mRNA. Patients and methods used are venous blood samples obtained from 30 NPC patients with biopsy-proven NPC and 20 healthy blood donors which were tested using a previously reported assay. Identity of products was confirmed by sequencing. Results obtained show that 10 of the 30 NPC were positive for CK19 transcripts in peripheral blood, suggesting the presence of tumour epithelial cells. No CK19 positive cells were detected in the 20 healthy volunteers. Nevertheless, we encountered the problem of CK19 pseudogene interference due to genomic DNA contamination of RNA preparations. Four of the 10 positive patients were proven to have CK19 mRNA, as assessed by sequencing. In the remaining six, the presence of the CK19 pseudogene may has masked the CK19 mRNA in RT-PCR and sequencing. In conclusion, we described an improved RT-PCR assay that is sensitive and has high clinical specificity to detect minimal metastatic disease in NPC patients. Nevertheless, one should be aware about the necessity to optimize the analytical specificity of the assay.

Key words: Nasopharyngeal carcinomas, micrometastasis, cytokeratin 19, nested reverse transcriptase polymerase.

INTRODUCTION

Nasopharyngeal Carcinoma (NPC) is a rare tumor arising from the mucosal epithelium of the nasopharynx. It is vastly more common in South East Asia and North Africa than elsewhere (Chen et al., 2013). The majority of NPC

Patients present at the moment of diagnosis have occult or detectable metastases. Indeed, NPC is difficult to detect early due to the anatomical position of the nasopharynx, its symptoms that mimic those of many other conditions and its tendency to present with cervical lymph node metastases. Distant metastases to the liver, the lung or the bone marrow and locoregional recurrence to the nasopharynx and the neck are the main causes of mortatlity and therapeutic failure (Chen et al., 2013). Surgical or radiotherapy treatment controls only the primary tumor and does not prevent the appearance of distant metastases treated only by chemotherapy. Chemotherapy treatment is little efficient and not well tolerated by the patient. So, early detection of micro-metastases and the prediction of recurrence will be an important issue in the prognostic of newly diagnosed NPC patients, in the choice of systemic treatment and in monitoring the effectiveness of the adjuvant therapy.

To improve on existing methods of detection, we evaluated, using a nested reverse-transcriptase polyme-rase chain reaction (RT-PCR) assay, the RNA transcript encoding for the intermediate filament protein cytokeratin 19 (CK19), as a marker for circulating nasopharyngeal carcinoma cells in the blood of patients with NPC. Since this class of cytokeratin is expressed only in normal and malignant epithelial cells but is absent in hematopoietic tissues of healthy subjects, detection of CK19 transcript in the peripheral blood of patients with known NPC should indicate the presence of circulating nasopharyn-geal carcinoma cells.

MATERIALS AND METHODS

Patients and tissue samples

The study population consisted of 30 non apparent patients with biopsy-proven NPC (25 previously untreated and 5 were treated at the time of sample collection) admitted at Salah Azaiez Oncology Institute of Tunis, Tunisia; before blood sampling, all NPC patients received detailed staging work-up. Patient characteristics are shown in Table 1. In addition, 20 healthy volunteers admitted as bone marrow donors in the National Center for Blood Transfusion of Tunis were tested as negative controls. Informed consent was obtained from all subjects.

Blood processing and RNA extraction

From each patient, 8 ml of peripheral blood were collected in 15 ml heparin- rinsed tube. Before blood collection, an initial 3 ml of blood was discarded to avoid possible contamination by normal skin cells. Whole blood was centrifuged at 4000 rpm for 15 min. The cellular pellet was then subjected to the RNA extraction procedure using the acid guanine-phenol-chloroform technique, with TRIzole (Invi-trogen), according to the manufacturer's instructions. After isolation, the RNA pellet was suspended in 50 µl diethyl-pyrocarbonate (DEPC)-treated water (0.01%) and stored at -20°C.

cDNA synthesis

0.6 µg of total cellular RNA was reverse-transcribed in a 25 µl reac-tion mixture containing 5 units of avian myeloblastosis virus reverse transcriptase (AMV) (Promega), 10 mM Tris/HCl (pH 8.3), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM spermidine, 2 mM dithiotreitol, 0.4 mM dNTP (Promega) and 25 µM oligo(dT)$_{18}$. The reaction was performed with a denaturing step of the RNA template (5 min at 90°C, snap cooled) followed by a synthesis step (1 h at 42°C). The reaction was terminated by heating at 92°C for 5 min.

PCR amplification of β-actin transcripts

In order to control the integrity of the extracted RNA and the quality of the first strand of the synthesized cDNA, all patients were tested for the presence of the β-actin RNA, a housekeeping gene expressed by all cells of the organism. The PCR mixture contained 5 µl of the cDNA from the reverse transcription, 20 pmol of each β-actin primers (sense: CACTGTGTTGGCGTACAGGT, anti-sense: TCATCACCATTGGCAATGAG), 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM dNTP and 2.5 units of Go Taq DNA polymerase (Promega) in a total volume of 50 µl. PCR was performed with the following conditions: initial denaturation at 95°C for 5 min, followed by 40 cycles at 94°C for 40 s, 54°C for 1 min, and 72°C for 50 s, followed by a final extension at 72°C for 10 min. The PCR product (expected size, 154 bp) was loaded on a 1.5% agarose gel.

PCR amplification of CK-19 transcripts

We evaluated the RNA transcript encoding for CK-19 as a marker for epithelial cells using a specific RT-PCR-based assay described by Lin et al. (2000). To increase the sensitivity of this assay, the primary amplified product was subjected to a nested PCR. In order to exclude amplification from the genomic DNA, both outer and inner primer pairs were selected from two different exons and one of the outer primer pairs was located across the junction of exon-intron (Figure 2). The first round of the PCR was performed using 5 µl of the cDNA as template. The PCR mixture contained 20 pmol of each CK-19 outer primers (sense: ACCATGAGGAGGAAATCAGTAC, antisense: ATCTTCCTGTCCCTCGAGCA), 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 10mM dNTP and 2.5 units of Go Taq DNA polymerase in a total volume of 50 µl. The PCR program included 1 cycle at 94°C for 5 min, and 40 cycles consisting of 40 s at 94°C, 1 min at 60°C and 50 s at 72°C. Finally, an extension at 72°C for 10 min. For the second round of amplification, 5 µl of the first round PCR product was added to 20 pmol of each CK19 inner primers (sense: CCAAGATCCTGAGTGACATGCGAAG, anti-sense: GATGTCCATGAGCCGCTGGTAC), 10 mM Tris/ HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM dNTP and 2.5 units of Go Taq DNA polymerase (Promega) in a total volume of 50 µl. The second PCR was carried out under the same conditions as the first PCR.

The PCR products were electrophoresed on a 2% agarose gel and analyzed by direct visualization under UV after ethidium bromide staining. The presence of a 518-bp first round product or a 371-bp second-round product indicated the presence of CK19 transcripts. All positive CK19 positive RT-PCR assays were repeated from a second aliquot of RNA. RNA extracted from two surgical biopsies of epithelial origin (mammary tumor and healthy skin) were used as positive controls. Mouse immunoglobulin hyper-variable region cDNA preparation was routinely processed as negative control.

Direct sequencing

To verify the identity of the PCR product, all positive signals were purified from agarose gel by QIAquick kit (Qiagen), according to the

Table 1. Clinical features of NPC patients and molecular data.

Patient N°	Sex	Age	Stage	TNM	Treatment	CK19 RT-PCR*	CK19 transcripts*
1	F	61	IVB	T4N3M0	No	+	+
5	F	21	IVA	T4N2M0	No	-	-
6	M	76	IVB	T4N3M0	No	+	+
8	M	66	IVB	T4N3 M0	No	-	-
10	M	66	NA	N0	No	-	-
11	M	52	IVA	T4N2cM0	No	-	-
12	M	56	IVB	T4N3M0	CT	+	-
14	M	66	IVC	M1	CT	+	-
15	F	49	IVA	T4N0M0	No	-	-
16	M	23	IVB	T4N3M0	CT+RT	+	-
18	F	68	IVA	T4N0M0	No	-	-
23	M	73	IVC	M1	CT	-	-
24	M	57	III	T3N2aM0	No	-	-
26	M	17	IVB	T3N3M0	No	-	-
27	F	57	NA	NA	No	-	-
28	M	42	NA	NA	No	-	-
29	M	58	NA	NA	No	+	-
30	M	16	IVA	T4N0M0	No	+	-
32	M	37	IVB	T4N3M0	No	-	-
34	M	42	III	T3N0M0	RT	-	-
36	M	42	NA	NA	No	-	-
37	F	49	IVB	T2N3M0	No	-	-
38	M	44	IVC	T3N3M1	No	-	-
40	M	NA	NA	NA	No	-	-
43	M	52	IVB	T3N3M0	No	-	-
45	M	NA	NA	NA	No	-	-
46	M	NA	NA	NA	No	+	+
47	M	47	IVC	T4N2aM1	CT+RT	+	-
48	M	NA	NA	NA	No	-	-
49	M	NA	NA	NA	No	+	+

*CK19 RT-nested PCR: +, presence of the expected 371-pb product; -, absence of the product; CK19 transcripts: +, presence of specific CK19 transcripts, as verified by sequencing; -, absence of CK19 transcripts detected by sequencing. CT, Chemotherapy; RT, radiotherapy; NA, not available.

manufacturer's instructions, and then sequenced using the inner primers and the BigDye™ Terminator v3.0 DNA sequencing kit with the ABI PRISM 3100-Avant Genetic Analyser (Applied Biosystems). Sequences were analysed using Basecaller Sequencing Analysis Software™ v3.7.

RESULTS

Clinical and pathological features

We studied a cohort of 30 Tunisian patients with non apparent but biopsy-proven NPC. Clinical characteristics and stage distribution of patients are summarized in Table 1. Probands enrolled in the study comprised 24 males and six females. The individual age at the moment of blood collection ranged from 16 to 76 with a median age of 46 years.

Histologically, all tumors were undifferentiated naso-pharyngeal carcinoma type (UCNT). Accor-ding to the adopted TNM classification (AJC/UICC 1986) (Bast et al., 2003), the stage distribution at the moment of blood collection of the 19 NPC patients for which medical records were available was as follows: early stage (2 stage III disease), advanced stage (13 stage IV A or IV B disease without distant metastasis, M0) and metasta-sized (4 stage IVC, M1). Among the five treated patients before sample collection, 2 had confirmed distant metas-tasis to liver or bone marrow, whereas three had loco-regional relapse to cavum and neck.

RNA extraction and reverse transcription

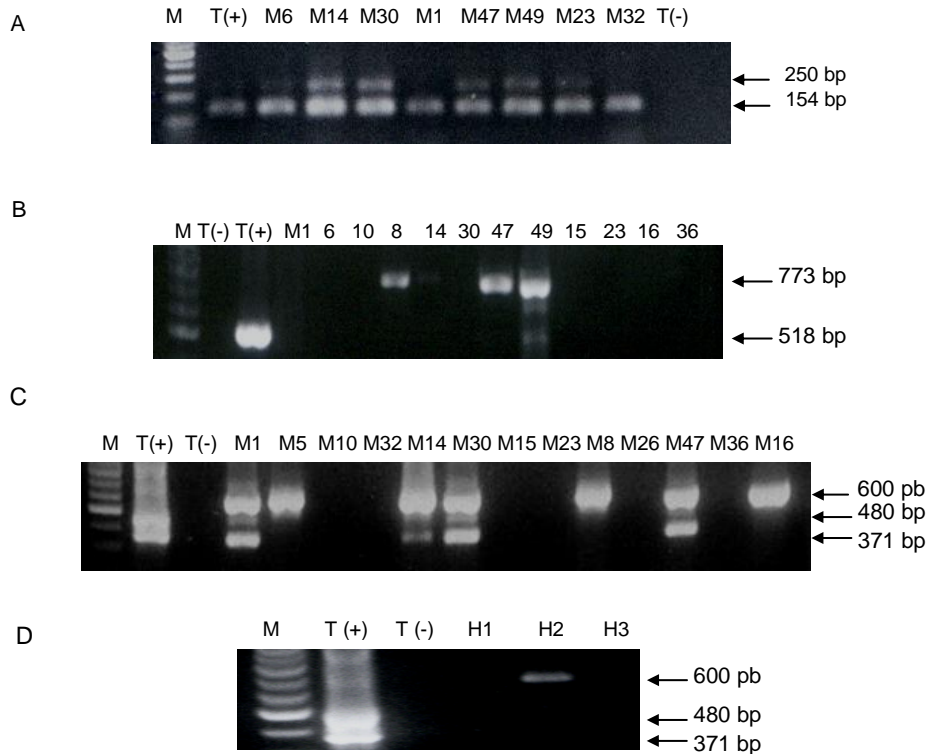Total RNA were extracted from 8 ml of peripheral blood

Figure 1. Results of RT-PCR for CK19 in NPC patients and controls. **A)** β-actin in NPC patients: The 154-bp product corresponds to β–actin cDNA. The 250-bp signal corresponds to amplification of contaminating genomic DNA. **B)** First round PCR for CK-19 in NPC patients: The expected 518-bp CK-19 PCR product is present only in the positive control [T(+), mammary carcinoma]. No product is detected in the blood of NPC patients (Mn). The 773-bp signal corresponds to contaminating genomic DNA.**C)** Second round PCR for CK19 mRNA in NPC patients. The expected 371-bp CK19 PCR product is present in the blood of several NPC patients (Mn), as well as in the internal positive control [T(+), mammary carcinoma]. The 480-bp product corresponds to RNA generated by alternative splicing. The 600-bp corresponds to contaminating CK19 genomic DNA. **D)** Second round PCR for CK19 mRNA in controls: The expected 371-bp CK-19 PCR product is present only in the positive control [T(+), mammary carcinoma]. No CK-19 PCR product is observed in the blood of healthy bone marrow donors (H1, H2 and H3). Healthy subject H2 showed the 600-bp contaminating genomic DNA band. M, 100 pb DNA marker (Promega); T(-), negative control (mouse immunoglobulin hypervariable region cDNA).

under RNase-free conditions using TRIzole. The first 3 ml of blood were discarded to ensure the exclusion of cutaneous epithelial cells in the samples, which normally express the CK19 gene. RNA was also extracted from two fresh biopsies of epithelial origin (mammary tumor and healthy skin) and run as positive controls for detection of CK19 transcripts using our RT-PCR assay. The first strand of the cDNA was synthesized under RNase-free conditions using the AMV reverse transcriptase.

Amplification of β-actin mRNA

All specimens from NPC patients, healthy subjects, healthy skin biopsy and mammary tumor biopsy showed appropriate amplification for β-actin transcripts (154-bp product), ruling out the presence of specimen degradation or inhibitory substances (Figure 1A). This result confirms the integrity of the RNA purified and the cDNA synthesized. Of note, an additional 250-bp product was shown with the expected 154-bp PCR product in some cases. This product was consistent with an amplification of the β-actin gene from genomic DNA contaminating some RNA preparations. This contamination was expected as no DNase treatment was included in our protocol in order to maximize the yield of RNA extracted from blood samples.

Detection of CK19 transcripts in controls

PCR assays for CK19 transcripts were tested first on cDNA obtained from reverse transcription of RNA extracted

from fresh healthy skin and mammary carcinoma biopsies. As expected, PCR amplification using CK19 outer primers and CK19 inner primers yielded a 518-bp fragment and a 371-bp fragment, respectively, both fragments corresponding to the CK19 cDNA, as verified by direct sequencing (Figure 1B and D). No PCR product was obtained from the cDNA of mouse immunoglobulin hypervariable region, confirming the specificity of the CK19 primers used (Figure 1B).

Detection of CK-19 transcripts in NPC patients and healthy blood donors

The expected 518-bp fragment at the first-round PCR was not detected in anyone of the healthy blood donors, nor NPC patients, including those having proved distant metastases to liver or bone marrow (Figure 1B). This was probably due to the rarity of epithelial neoplastic cells in the blood circulation at the moment of blood sample. This result shows the limit of the PCR sensitivity and confirms the necessity of a second round PCR. Of note, a 773-bp product was detected in 10 of the 30 tested NPC patients and in 1 of the 20 healthy blood donors (Figure 1B). This product corresponded to genomic DNA contaminating our RNA preparations as assessed by sequence alignment with the genomic sequence of CK19 (Genebank accession number NM_002276). The expected 371-bp fragment at the second-round PCR was detected from PCR products of the first PCR reaction of 10 of the 30 tested NPC patients (Table 1 and Figure 1C). In contrast, no product was detected in blood samples from the 20 healthy blood donors (Figure 1D). A 600-bp product was observed in the blood samples of 17 of the 30 NPC patients and in 1 of the 20 healthy blood donors analyzed, that corresponded to genomic DNA contamination, as assessed by sequence analysis.

Control of the specificity of the CK19 product by sequencing

The use of CK19 as a target gene in the detection of circulating tumor cells is limited by the interference of CK19 pseudogene in RT-PCR (Ruud et al., 1999). Indeed, CK19 pseudogene sequence (Genebank accession number U85961) shows a high degree of identity with the CK19 mRNA (84.7%) which may lead to the amplification of a 371-bp fragment from this pseudogene by the inner primer CK19-3. The comparison of the sequence of the 371-bp fragment to the CK19 cDNA (Genebank accession number NM_002276) and to the pseudogene (Genebank accession number U85961) revealed that this product corresponded to pseudogene in 6 of the 10 positive patients, that is, 60% false positive rate. In the four remaining cases (M1, M6, M46, M49), the 371-bp product showed 100% identity with the CK19

cDNA. Thus, sequencing showed that the used nested RT- PCR assay may lead to false positive cases due to pseudogene amplification. In other hand, this amplification may also mask the expression of CK19 mRNA giving false negatives by direct sequencing.

DISCUSSION

Metastatic dissemination is a key event in the history of cancerous pathology because it transforms a locoergional disease curable by local treatment, surgery and/or radiotherapy, to a widespread cancer whose treatment is complex and unpredictable.

Biological heterogeneity of neoplastic cells in the primary and secondary tumors is the principal cause of treatment failure of metastases. This highlights the importance to understand the molecular process of metastase and the necessity to establish a synoptic table of markers which allow easy prediction, detection and localization of active metastasis and finally identification and definition of therapeutic targets. Detection of circulating neoplastic cells by RT-PCR is principally based on selective amplification of genes that are exclusively expressed by these cells, but not expressed by normal blood cells. RNA being instable in extracellular environment, its detection indicates the presence of circulating living neoplastic cells in the examined blood. Many tumor- or organ- specific genes have been reported such as PSA for prostate carcinoma (Yates et al., 2012), mammaglobin and CK-19 for breast cancer (Chen et al., 2010) and CEA for gastro-intestinal (Zhu et al. 2012), breast cancer (Chen et al., 2010) as well as for colorectal cancer (Jones et al., 2013). However, these tumor- or organ specific markers still unknown for many solid tumors such as the case of NPC. For this reason, detection of micrometastasis often targets epithelial genes implicated in tissue differentiation. Since cytokeratin 19 is not expressed by any normal hematopoietic tissue, it has been reported to be the useful marker of occult metastasis to the peripheral blood, bone marrow and lymph nodes of patients with cancer of epithelial origin. Cytokeratins form a subgroup of intermediate filament protein predominantly located within cells of epithelial origin (Wittock et al., 2000).

In addition to a limited number of epithelial tissues, CK19 expression had also been found in colon carcinoma, small-cell and non-small-cell lung cancer, and prostate cancer. These findings potentially extend the utility of CK19 RT-PCR as a detection assay for a broad-spectrum of common malignancies. The aim of the present report was to evaluate the CK19 RT-PCR procedure for detecting occult metastasis in NPC, based on previously published assays. Using RNA extracted from two epithelial tissues (healthy skin biopsy and mammary carcinoma) as positive controls and the mouse immunoglobulin hypervariable region cDNA as negative
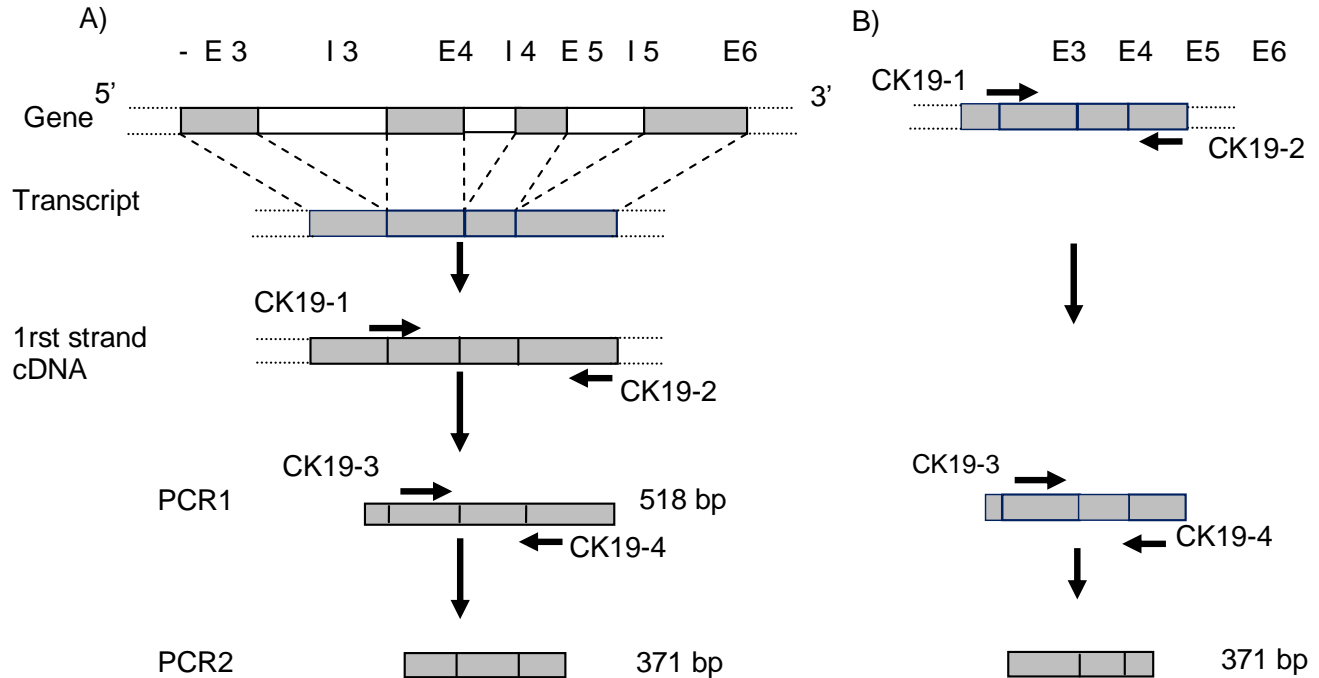
Figure 2. Schematic representation of the CK19 gene and CK19b pseudogene.
The CK19 gene (A) and CK19b pseudogene (B) are shown with location of introns (I) and exons (E), as well as the location of outer and inner primers and the size of the expected PCR products.

controls of RNA purification and RT-PCR, as well as the blood of 20 healthy bone marrow donors as negative controls for specificity of the assay, we could detect the presence of the CK19 transcript (371-bp product) in epithelial tissues and its absence in the blood of all the healthy subjects. These results highlight the specificity of expression of CK19 in epithelial tissue. The specificity was also checked by sequencing of the PCR product. According to our results and those reported by Lin et al. (2000), the RT-nested PCR assay seemed specific to detect the presence of CK19 mRNA in the blood. We have then applied this technique to 30 Tunisian NPC patients with histologically proven NPC in different stages. Positive results were obtained for 10 of them after a second round PCR. The first round of PCR was negative for all the blood samples and the expected product (518-pb) was seen only for the skin biopsy. This is probably due to the scarcity of disseminated tumor cells in peripheral blood when sampling. Because the assay published by Lin et al. (2000) appeared to use specific primers for CK19, we used their exact PCR conditions and nested primer sets for our study. However, in our samples, we were not able to reproduce the specific bands published by Lin et al. (2000). In our hands, the same primer set gave rise to CK19 pseudogene derived products in most cases, indicating the low robustness of the assay.

In the present study, we encountered several difficulties in using CK19 as target marker. Problems we encoun-

tered were primarily due to the interference of the CK19 pseudogene and to the unavoidable contamination of the RNA preparations by genomic DNA. Indeed, Ruud et al. (1999) have detected by RT-PCR a new pseudogene showing high homology to CK19 gene sequence (84.7%) except three small deletions and some punctual mutations. In our assay, pseudogene amplification gives a 371 bp fragment that is analogous to the product of amplification of the normal CK19 cDNA, with 73% of sequence similarity (27 nucleotide variations between the two products). To confirm the origin of the 371 bp fragment (gene or pseudogene), the nested PCR product of the 10 positive samples were sequenced. The comparison to reference sequences of CK19 cDNA (Genebank accession number NM_002276) and CK19 pseudogene (Genebank accession number U85961) revealed that the PCR product of only four of the ten positive samples corresponded to the CK19 cDNA. The sequence of the six remaining patients was identical to that of the CK19 pseudogene. According to the literature (Goeminne et al., 2000), genomic DNA and subsequent pseudogene contamination is frequent in RNA preparations. Its presence could interfere and mask the CK19 transcript in RT-PCR and sequencing. In order to improve the specificity of the reaction, we suggest designing new primers which will amplify specifically the CK19 cDNA.

Notably, only 2 of the 4 NPC patients with known distant metastasis were positive for the 371-bp fragment,

indicating false negative results. This result was probably due to the absence or the rarity of neoplastic cells in the analyzed samples. In conclusion, we have described an improved RT-PCR assay that is sensitive and has high clinical specificity to detect minimal metastatic disease in patients with NPC. Nevertheless, one should be aware about the necessity to optimize the analytical specificity of the assay. Whatever the case, in its present form, the CK19 RT-PCR nested assay allowed identification of specific CK19 transcripts in the blood of at least 4 NPC patients. This highlights the potential interest of this method for the detection of subjects with subclinical metastasis. This assay will also facilitate the selection of patient suitable for adjuvant chemotherapy in order to eradicate micrometastasis early and enhance the cure rate of cancer.

ACKNOWLEDGEMENTS

REFERENCES

Bast RC, Kufe DW, Pollock RE, Weichselbaum RR, Holland JF, Fre E (2000). Cancer medicine; 5 th edition. Section 27. Neoplasms of the Head and Neck cancer treatment. Table 86.23. TNM classification and stage grouping of the nasopharynx. (NCBI)

Chen MY, Jiang R, Guo L, Zou X, Liu Q, Sun R, Qiu F, Xia ZJ, Huang HQ, Zhang L, Hong MH, Mai HQ, Qian CN (2013). Locoregional radiotherapy in patients with distant metastases of nasopharyngeal carcinoma at diagnosis. China J. Cancer 32:604-613.

Chen Y, Zou TN, Wu ZP, Zhou YC, Gu YL, Liu X, Jin CG, Wang XC (2010). Detection of cytokeratin 19, human mammaglobin, and carcinoembryonic antigen-positive circulating tumor cells by three-marker reverse transcription-PCR assay and its relation to clinical outcome in early breast cancer. Int. J. Biol. Markers 25:59-68.

Goeminne JC, Guillaume T, Symann M (2000). Pitfalls in the detection of disseminated non-hematological tumor cells. Ann. Oncol. 11:785-792.

Jones C, Badger SA, Epanomeratikis E, McKie LD, Diamond T, Taylor MA (2013). Role of carcinoembryonic antigen as a marker for colorectal liver metastases. Br. J. Biomed. Sci. 70:47-50.

Lin JC, Tsai CS, Wang WY, Jan JS (2000). Detection of circulating tumor cells in venous blood of nasopharyngeal carcinoma patients by nested reverse transcriptase polymerase chain reaction. Kaohsiung J. Med Sci. 16:1-8.

Ruud P, Fodstad O, Hovig E (1999). Identification of a novel CK19 pseudogene, interfering with RT-PCR assays in the detection of circulating tumor cells. Int J Cancer. 80:119-125.

Wittock NV, Eady RA, McGrath JA (2000). Genomic organization and amplification of the human keratin 15 and keratin 19 genes. Biochem Biophys. Res. Commun. 267:462-5.

Yates DR, Rouprêt M, Drouin SJ, Comperat E, Ricci S, Lacave R, Sèbe P, Cancel-Tassin G, Bitker MO, Cussenot O (2012). Quantitative RT-PCR analysis of PSA and prostate-specific membrane antigen mRNA to detect circulating tumor cells improves recurrence-free survival nomogram prediction after radical prostatectomy. Prostate 72:1382-1388.

Zhu YB, Ge SH, Zhang LH, Wang XH, Xing XF, DU H, Hu Y, Li YA, Jia YN, Lin Y, Fan B, Ji JF (2012). Clinical value of serum CEA, CA19-9, CA72-4 and CA242 in the diagnosis and prognosis of gastric cancer. Zhonghua Wei Chang Wai Ke Za Zhi.15:161-164.