High False-Positive Rate of Cytokeratin-19 in Detecting Circulating Tumor Cells for Nasopharyngeal Carcinoma

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Background: Nasopharyngeal carcinoma (NPC) harbors a higher metastatic potential than other head and neck cancers. In order to seek a possible surrogate marker for early detection of recurrent or metastatic disease, we tested the feasibility of cytokeratin-19 (CK-19)-nested reverse-transcription polymerase chain reaction (RT-PCR) for detecting circulating tumor cells in NPC patients.

Methods: Two tubes of blood were sequentially collected in individual draws from 7 NPC patients and 15 healthy persons. Total ribonucleic acid (RNA) was extracted from blood cells and treated with deoxyribonuclease. The RNA was then subjected to RT and nested PCR with specific CK-19 primers. The reaction products were run on an agarose gel and visualized under UV light. The sequences of the products were determined using an ABI377 automatic sequencer.

Results: Among the 7 NPC cases, 4 cases presented CK-19 expression with 2 in both tubes, 1 in the first tube, and 1 in the second tube. In the control group, 8 of 15 cases also presented CK-19 expression with 6 in both tubes and 2 in the second tube resulting in a 53.3% false-positive rate. Incidentally, an aberrant splicing product lacking exon 4 of CK-19 messenger RNA was discovered.

Conclusion: Results of the present study indicate that the CK-19-nested RT-PCR is not suitable for detecting circulating tumor cells in NPC patients because of a high false-positive rate in the control group. The reason for the high rate of false-positives may be attributed to pseudogenes, different blood cell separation methods, or illegitimate expression of CK-19 in blood cells.
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Key words: nasopharyngeal carcinoma, cytokeratin-19, circulating tumor cells.

Nasopharyngeal carcinoma (NPC) is a distinct form of head and neck cancer. Many features of its epidemiology, etiology, histopathology, presentation, and prognosis differ from malignancies occurring at other sites of the head and neck.[1] According to the Cancer Registry Annual Report of the Republic of China 1997, the incidence of NPC in Taiwan was 7.90/100,000 in men and 2.96/100,000 in women, and the median age of occurrence was 48 years.[2] It ranked 12th in cancer mortalities in Taiwan in the year 2000.[3] Therapy of early-stage NPC has been very successful. Treatment group in the Koo Foundation Sun Yat-Sen Cancer Center has achieved 3-year disease-free survival of 91.7% in the radiation arm and 96.9% in the concurrent chemoradiotherapy arm for stage I and II diseases (according

to the AJCC 1997 staging system).[4] However, the outcome of locally advanced disease is not so satisfactory. The same group reported 3-year disease-free survival of 87.7% in stage III and 51.9% in stage IV disease.[5] Distant metastasis constitutes the main pattern of treatment failure for locally advanced NPC. Intergroup study 0099 reported a distant metastasis rate of 61% for the radiation arm and 23% for combined radiation and chemotherapy arm.[6] A Hong Kong group reported a greater than 50% distant metastasis rate in either the radiation or combined modality arm.[7] These high-risk patients may require different treatment protocols to achieve better results. To identify this subgroup, it is mandatory to find a surrogate marker for future distant metastasis.

Recently, detection of cancer cells by amplification of cancer-related genes in peripheral blood or tissue fluid has become a feasible method to detect minimal residual disease. Such tumor markers include prostate-specific antigen (PSA) in prostate cancer, carcinoembryonic antigen (CEA) in colon cancer, and squamous cell carcinoma antigen (SCC) in cervical cancer.[8-10] Cytokeratin is an intermediate filament of epithelial cells and has been suggested as a marker for the sensitive detection of circulating epithelial cancer cells by reverse-transcription polymerase chain reaction (RT-PCR).[11,12] Cytokeratin-19 (CK-19), a member of the cytokeratin family, has been successfully used to detect circulating cancer cells in breast cancer and lung cancer.[13,14] Lin et al. have demonstrated an application of CK-19-nested RT-PCR to detect circulating tumor cells in NPC patients.[15] The aim of the present study was to test whether the application can be reproduced by a different system of CK-19-nested RT-PCR.

METHODS

Peripheral blood was collected from NPC patients and normal healthy persons. To prevent skin epithelial cell contamination, 2 samples were collected sequentially in each blood withdrawal from each subject, with 2 ml in the first tube and another 2 ml in the second tube. The first tube of blood acted as a control, and the second tube of blood acted as the experimental sample. The blood was placed in an ethylenediamine tetra-acetic acid-containing tube and kept in an ice bath. An NPC cell line (NPC-117-78), kindly provided by Prof. Chin-Tang Lin

(Department of Pathology, National Taiwan University Hospital) was used as a positive control.

Ribonucleic acid (RNA) was extracted from peripheral blood using a QIAamp RNA blood mini kit (Qiagen) following instructions provided by the manual. RNA was finally eluted with 30 µl of ribonuclease-free water, treated with RNA free-deoxyribonuclease (Promega), and stored at -20°C. The quality of RNA was tested by amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA.

Reverse transcription was carried out using a First Strand cDNA synthesis kit (Amersham Pharmacia Biotech). The RNA was denatured at 65 °C for 10 min, then chilled on ice. The reaction mixture was made up of 8 µl of RNA, 5 µl of Bulk First-Strand cDNA reaction mix, 1 µl of dithiothreitol solution, and 1 µl of NotI-d(T)18 primer. The reaction mix was incubated at 37°C for 1 h then stored at -20°C.

The nested PCR was based on the protocol of Datta et al.[13] with some modification. Each RT-PCR reaction was accompanied by a negative control of a PCR-only reaction. The reaction was performed in a volume of 20 µl consisting of 1 µl of RT reaction solution, 2 µl of 10x PCR buffer, 1.5 mM $MgCl_2$, 100 mM dNTP, 1 U Taq polymerase (MDBio), and 100 ng primers. The PCR amplification was carried out in a thermal cycler (PTC-100, MJ Research) under the following conditions: 1 cycle of 95°C for 5 min; 5 cycles of 95°C for 30s, 65°C for 30s, and 72°C for 2 min; 20 cycles of 95°C for 30s, 67°C for 30s, and 72°C for 2 min; and then 1 cycle of 72°C for 5 min. The outer primers were 5'-AAGCTAACCAT-GCAGAACCTCAAC GACCGC and 5'-TTATT-GGCAGGTCAGGAGAAGAGCC, and the inner primers were 5'-TCCCGCGACTACAGCCACTAC-TACACGACC and 5'-CGCGACTTGA TGTCCAT-GAGCCGCTGGTAC. As described by Datta et al., the 3' primer was designed to preclude transcription of a pseudogene.[13] The reproducibility was examined by repeating the nested RT-PCR. PCR samples were run on a 1% agarose gel containing ethidium bromide, and the band was visualized under UV light.

PCR products were recovered using a Geneclean III kit and were cloned into pGEM-T using the pGEM-T vector system (Promega). Sequences of the PCR products were determined

using an ABI377 automatic sequencer following the instructions provided by the manual.

Data were analyzed for statistical significance using the chi-square test.

RESULTS

Peripheral blood was sampled from 7 NPC and 15 healthy controls, and the respective RNA was extracted. The stages of the 7 NPC patients included 1 case of stage III, 2 cases of stage IVA, and 4 cases of stage IVC (AJCC, 1997 staging system). The quality of RNA was tested by RT-PCR of GAPDH messenger RNA (mRNA). All RNA samples produced the expected bands of 983 bp in length. The NPC cell line produced the expected positive control band of 745 bp in length. In the control group, 8 subjects (53.3%) produced the bands, with 6 in both tubes of blood and 2 only in the second tube of blood (Fig. 1). In the 7 NPC cases, 4 (55.6%) produced the bands, with 2 in both tubes, 1 in the first tube, and 1 in the second tube (Fig. 2). The repeated experiment
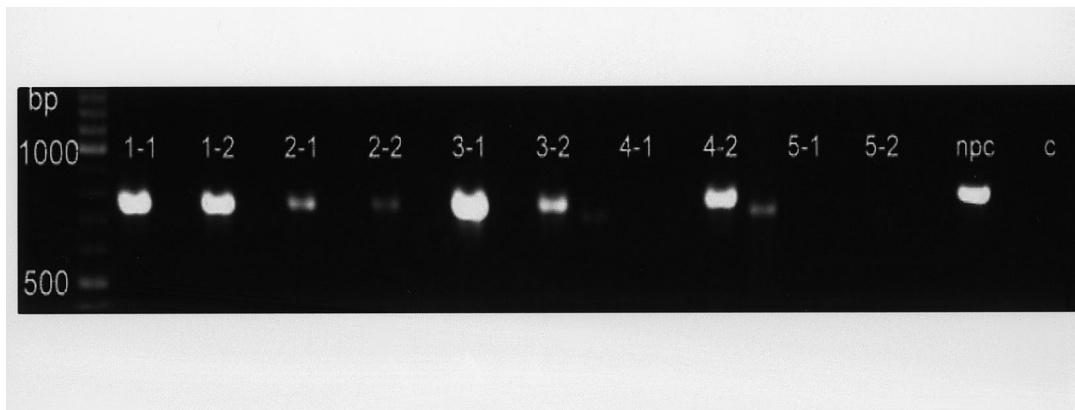
Fig. 1 Control group of nested RT-PCR of CK-19 mRNA. 1-1, 2-1, 3-1, 4-1, and 5-1 are the first tubes of blood samples of control cases 1, 2, 3, 4, and 5; 1-2, 2-2, 3-2, 4-2, and 5-2 are the second tubes of blood samples of control cases 1, 2, 3, 4, and 5; npc is the NPC 117-78 positive control; c is the no RT input negative control. The lane to the right of each RT-PCR, except c, is the PCR-only reaction of the negative control for each respective RT-PCR. Note that the CK-19 band of 745 bp appeared in 1-1, 1-2, 2-1, 2-2, 3-1, 3-2, 4-2, and npc RT-PCRs. There was a slightly shorter band which appeared in the PCR-only reactions of 3-2 and 4-2.
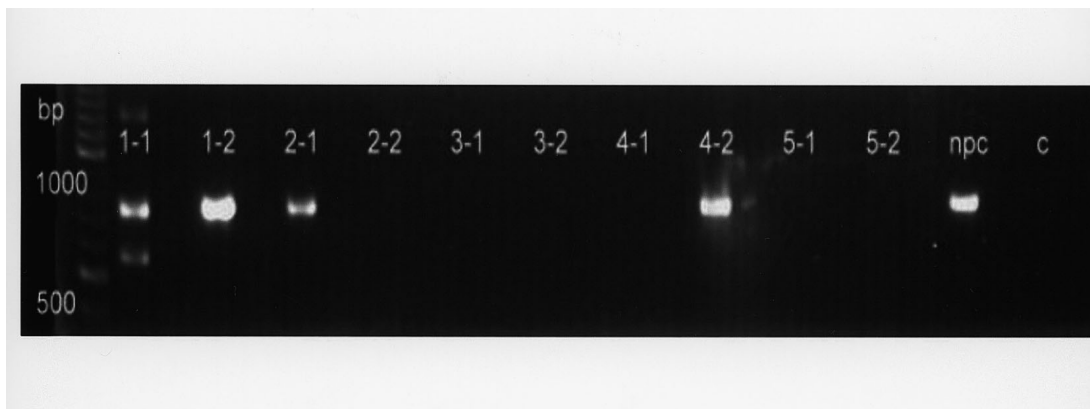
Fig. 2 NPC group of nested RT-PCR of CK-19 mRNA. 1-1, 2-1, 3-1, 4-1, and 5-1 are the first tubes of blood samples of NPC patients 1, 2, 3, 4, and 5; 1-2, 2-2, 3-2, 4-2, and 5-2 are the second tubes of blood samples of NPC patients 1, 2, 3, 4, and 5; npc is the NPC 117-78 positive control; c is the no RT input negative control. The lane to the right of each RT-PCR, except c, is the PCR-only reaction of the negative control for each respective RT-PCR. Note that the CK-19 band of 745 bp appeared in 1-1, 1-2, 2-1, 4-2, and npc RT-PCRs. An aberrant splicing product lacking exon 4 occurred in 1-1 RT-PCR.

demonstrated similar results. There was no statistical difference between the 2 groups as to the rate of PCR product ($p > 0.1$). The PCR products were then cloned and sequenced. The sequence was confirmed to be the CK-19 mRNA (accession no.: XM_031433). Two novel bands were observed. One, slightly shorter than the CK-19 product (695 vs. 745 bp), appeared in 2 PCR-only reactions in 2 control subjects (Fig. 1) the sequence of which revealed that it is a newly discovered CK-19 pseudogene (accession no.: U85961).[16] The other one appeared below the CK-19 band in the RT-PCR reaction from the first tube of NPC patient 1 (564 bp in length) (Fig. 2). Aligning the sequence with the CK-19 mRNA and mapping the sequence to the CK-19 gene[17] indicated that it is a product of aberrant splicing of the CK-19 gene in which exon 4 is spliced out.

DISCUSSION

In the report of Lin et al., the authors demonstrate that the increased positive detection rate of CK-19 mRNA in peripheral blood corresponded to greater clinical stages of NPC patients.[15] There were no false positives in that study. This result could not be reproduced in the present study using a different system of CK-19-nested RT-PCR because of the high rate of false positives in the control group. The high rate of false positives in our study (53.3%) contrasts with that of the original paper on this method in which the false-positive rate was only 2.6%. In a review of the literature, several investigators have reported low false-positive rates using the same method, while others obtained high false-positive rates as we did (Table 1).[13,14,18-21] Discrepancies in false-positive rates among investigators have also been observed in other systems for detecting circulating tumor cells, such as CK-18, CEA, and PSA.[22]

An obvious question arises concerning these discrepancies among different studies in the literature.

In the present study, we found that a possible source of false positives might arise from amplification of a contaminated pseudogene, which was discovered recently by Ruud et al.[16] This pseudogene has a high degree of identity with CK-19 mRNA but because of a termination codon at base 496, it has a shorter amplified product of 695 bp in length. In Datta's original report, primers were designed such that only CK-19 mRNA was amplified but not the then-known pseudogene published by Savtchenko et al.,[23] but these primers could not discriminate between the newly discovered pseudogene and CK-19 mRNA.[16] The authors suggested that previous work with CK-19 as a marker should be reassessed in view of the existence of the new pseudogene. However, because it is not difficult to detect the size difference between the amplified CK-19 mRNA and the pseudogene and most negative controls of the PCR did not produce the band, we believe that the contaminating pseudogene was not a chief source of false positives in the present study.

Another possibility is the different methods used for blood cell separation. A common way to separate blood cells is by Ficoll density-gradient centrifugation, which predominantly recovers mononuclear cells such as lymphocytes and monocytes while removing granulocytes. In contrast, the erythrocyte-lysis method, which was applied in the kit we used, recovers all nucleated cells including granulocytes. It is conceivably possible that granulocytes but not lymphocytes or monocytes express CK-19, leading to high rates of false positives in studies employing the erythrocyte-lysis method. Although Peck et al. described how epidermal cell contamination during peripheral blood drawing is the main source of false positives and can be excluded by a 2-step blood sam-

Table 1. Incidence of False-positive Rates in the Control Group by Datta's Protocol of CK19-nested RT-PCR

Authors	Year of publication	False-positive rate (%)	Reference
Datta et al.	(1994)	2.6	13
Krismannet et al.	(1995)	38	18
Peck et al.	(1998)	1.6	14
Yeh et al.	(1998)	not mentioned	19
Eltahir et al.	(1998)	high	20
Berois et al.	(2000)	0	21
Kao et al.	(2001)	53.3	present study

pling procedure,[14] our results did not support this notion because the CK-19 band appeared in both tubes of blood samples in most cases. These data suggest the evidence of CK-19 expression in certain cellular constituents in blood.

Some authors have hypothesized that illegitimate expression can be the cause of a high false-positive rate.[22] The possible low-level transcription of tissue-specific genes in non-specific cells may reflect the general process of illegitimate transcription.[24] The existence of such transcripts has been documented for 17 different genes by RT-PCR and has proven useful in the analysis of a number of genetic disorders. Although the abundance of these transcripts in inappropriate cells is very low, estimated at 1 mRNA molecule per 100-1000 cells, [24] it is considered possible that the high sensitivity of nested RT-PCR, at 1 in 10^7 cells,[14] may still be capable of detecting such a low level of transcript illegitimately expressed by blood cells, leading to high rates of false positives. In the current study we incidentally discovered an aberrant splicing of the CK-19 gene in which exon 4 is spliced out. The significance of this phenomenon remains to be investigated.

Taken together, we conclude that under the present experimental conditions, a high rate of false positives was observed, and this caused us to adopt a conservative attitude in applying the system to detect circulating epithelial tumor cells in NPC patients. Recent technical advances in molecular biology may produce a new strategy for the selection of more suitable target genes such as differential display, SAGE, and microarray.[25] In theory, it should be possible to efficiently screen for genes that are highly expressed in certain tumors but not in the cellular constituents of blood or bone marrow. We believe that the future of RNA-based methods for detecting circulating tumor cells depends on the identification and validation of appropriate panels of suitable target genes.

Acknowledgments

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周邊血液細胞角質素-19偵測鼻咽癌病人癌細胞之高僞陽性

高瑞和　黃立志

背 景： 鼻咽癌相較於其他頭頸部腫瘤，具有較高程度的遠處轉移能力。爲了尋找一可能替代指標以早期發現疾病復發或轉移，我們測試細胞角質素-19之巢式反轉錄－聚合脢連鎖反應系統是否可用來偵測鼻咽癌病人週邊血液的癌細胞。

方 法： 爲了避免在採血過程中檢體受到皮膚上皮細胞的干擾，我們採取連續兩段式抽血方式。從7位鼻咽癌病人及15位正常人之血液細胞中萃取RNA並以DNase處理過。利用這些RNA以及特殊的細胞角質素-19引子來施行巢式反轉錄－聚合脢連鎖反應。將反應產物跑在膠片上並以紫外線光檢視結果。反應產物以自動定序機定序。

結 果： 在7位鼻咽癌病人當中，有4位 (2位在兩段血、1位在第1段血、1位在第2段血) 有細胞角質素-19的表現。但是在15位正常人中也有8位 (6位在兩段血、2位在第2段血) 也有細胞角質素-19的表現，導致53.3%的僞陽性。實驗中意外發現少了第四exon的細胞角質素-19異常splicing 產物。

結 論： 本實驗顯示高僞陽性致使細胞角質素-19之巢式反轉錄－聚合脢連鎖反應系統不適合應用在偵測鼻咽癌血液中的癌細胞。這種無特異性的原因可能來自於假基因，不同的血球分離術，或者是細胞角質素-19基因本身在血球細胞的非法性表現。

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關鍵字： 鼻咽癌，細胞角質素-19，週邊血液癌細胞。