

DETECTION OF CYTOKERATIN 19 MRNA IN BLOOD AS AN EARLY MARKER OF MICROMETASTATIC TUMOR CELLS OF NASOPHARYNGEAL CARCINOMA PATIENTS IN SURABAYA

Hariato Notopuro¹, Widodo A Kentjono², Retno Handajani¹, Francisca Notopuro³

¹Departement of Biochemistry, Airlangga University School of Medicine and Institute of Tropical Disease

²Departement of Ear, Nose and Throat, Airlangga University School of Medicine, Dr Soetomo Teaching Hospital

³Departement of Radiology, Airlangga University School of Medicine, and Dr Soetomo Teaching Hospital

ABSTRACT

Nasopharyngeal carcinoma (NPC) is a unique epithelial malignancy triggered by Epstein Barr virus that occurs at a high frequency in certain regions of southeast Asia. Previous studies reported that this tumor was a radiosensitive but the predominant failure after adequate radiotherapy was due to its distant metastasis. It would be of value to detect the presence of metastasis as early as possible. Previous studies have shown that cytokeratin 19 (Ck 19) positive cells indicate the presence of micrometastasis. The purpose of this study was to evaluate the presence of Ck 19 mRNA by nested RT-PCR in established NPC patients and its correlation to the various stages of NPC as determined by conventional clinical examination. The reported sensitivity of RT-PCR was around $1/10^7$, i.e one cancer cell can be detected among 10^7 normal blood cells. Venous blood samples from 30 patients with biopsy-proven nasopharyngeal carcinoma and 10 ENT non NPC patients were tested. After we isolated total mRNA with Trizol reagent, the procedure was continued with nested RT-PCR. The primer sets were directed to conserved regions of Ck 19 genome encoding cytokeratin protein (outer primers: Ck 19-1 & Ck 19-2 and inner primers: Ck 19-3 & Ck 19-4). A distinct 518 bp band (it cannot be seen on agarose gel electrophoresis) and 371 bp of the PCR products indicated the presence of mRNA Ck 19. Results showed that Ck 19 mRNA was obtained in 22 (73%) of 25 NPC patients with several stages and none of 10 (0%) ENT non NPC patients. We divided 30 NPC patients into 4 groups according to TNM system (WHO criteria): Stage I: none, Stage IIa : 1 patient (Ck 19 mRNA was negative), Stage IIb: 1 patient (Ck 19 mRNA was negative), Stage III: 3 patients from 5 patients had Ck 19 mRNA positive (60%), Stage IVa: 5 patients from 7 had Ck 19 mRNA positive (72%) and Stage IVb: 14 patients from 16 patients had Ck 19 mRNA positive (87,5%). No Ck 19 positive cells were detected in control group, 10 patients ENT non NPC (0%), i.e 100% specificity. Our data indicated that the positive detection rate for Ck 19 mRNA in peripheral blood increased as the clinical stage of tumor increased, and the correlation was strong with statistical significance of $r = 0.95$ and $t > 0.02$. In conclusion, this study shows that the presence of Ck 19 mRNA, as detected by RT-PCR in NPC, is only significantly associated with micrometastasis tumor. It is a highly specific and sensitive method. Ck 19 mRNA detection is a useful and reliable method to monitor local recurrence and tumor metastasis in NPC patients. It helps to detect recurrence and tumor metastasis early and may improve local and systemic control and enhance patient's survival.

Keywords: Nasopharyngeal Carcinoma, Ck 19 mRNA, nested RT-PCR, micrometastatic tumor cells

Correspondence: Harianto Notopuro, Departement of Biochemistry, Airlangga University School of Medicine, Jl. Prof Dr Moestopo 47, Surabaya, phone 62-31-5020251 ext 140.

INTRODUCTION

Nasopharyngeal cancer (NPC) is a malignancy involving the epithelial cells, whose dispersion is influenced by geographical factors. Low incidence rate of this disease is found in Europe, North America, Japan, and India. Higher incidence rate is found southern China, Hongkong, Alaska and Greenland, while the moderate incidence rate can be found in northern China, North Africa, and Southeast Asia, including Indonesia (Lanier et al. 1980; Muir 1971). In Indonesia, NPC is the fifth malignant cancer from then most common types of cancer found in men and women (1999), and

comprised 5.78% of all cancerous tumors found. Previous studies reported that Epstein Barr virus (EBV) has a close and strong association as the cause of NPC and Burkitt's Lymphoma, supported by the finding of EBV genome in NPC tumor tissue and in peripheral blood of NPC patients based on PCR examination (Lin et al. 2001; Lin et al. 2004).

NPC can be regarded as unique head and neck tumor that has several different characteristics to the other head and neck malignancies. Radiation therapy is a treatment of choice for this tumor, due its radiosensitive nature and the obstacle of its anatomic site for having

surgical procedure. The result of NPC treatment in early stage provides satisfactory result. However, since most of patients come for treatment at advanced stage, the result of radiation therapy becomes less satisfactory. The failure of radiation therapy results in local tumor recurrence and distant metastasis with poor prognosis since the first two year survival is less than 10%. The median survival time < 6 months for untreated patients and about 12 months for patients who receive chemotherapy only (Hao et al. 2004; Kuo et al. 1996). Recently, a number of reports indicate that the detection of micrometastasis using PCR (for a specific cancer gene sequence) is more sensitive than other conventional clinical methods such as X-ray observation, tomography, CT scan and even the MRI. Therefore, the advantage of this method is, in addition to more accurate tumor staging determination, to assist the follow-up of the therapy that finally improve the prognosis of the NPC patients.

World Health Organization (WHO) classifies NPC based on its differentiation degree into WHO-1 : Keratinizing squamous cell carcinoma (10%), highly differentiated with features of epithelial growth and keratine filaments; WHO-2 : Non keratinizing carcinoma (5%), whose feature is still demonstrating epithelial cell growth; and WHO-3 (85%) : Undifferentiated carcinoma (UNPC), in which keratine is absent with irregular epithelial cell growth. Patients with NPC WHO-2 and WHO-3 presenting with the increase of IgG and IgA levels against VCA and EA, while WHO-1 Ca patients have IgG and IgA levels against ABV almost similar to that of normal control (Henle et al. 1973, Hu et al. 1996).

NPC classification based on T, N, M system of the Union Internationale Centre of Cancer (1986) is as follows: T = condition of primary tumor, its size and extension. T1: limited to nasopharynx. T2: extended to oropharynx and/or nasal fossa. T2a: without extension to parapharynx. T2b: with extension to parapharynx. T3: invasion to bone structure and/or paranasal sinus. T4: extend intracranially and/or involving cerebral nerve, intratemporal fossa, hipopharynx and orbita. N indicates the condition of regional lymph nodes, in which N0: no tissue enlargement, N1: ipsilateral tissue enlargement < 6 cm, N2: bilateral tissue enlargement < 6 cm, N3: tissue enlargement > 6 cm or supraclavicular extension. M indicates distant metastasis. M0: no distant metastasis, M1: presence of distant metastasis. NPC classification according to its stage is as follows: I:T1, N0, M0, IIA :T2a, N0, M0, IIB :T1, N1, M0, T2a, N1, M0, T2b, N0-1, M0, III : T1-2, N2, M0, T3, N0-2, M0, IVA :T4, N0-2, M0, IVB : Each T, N3, M0, and IVC : Each T, each N, M1.

NPC is rarely found in western countries. Its incidence rate is only 0.25% of all types of malignant cancer. Of interest is the NPC incidence among Indian and Chinese populations who live in Singapore. Both populations are together exposed to EBV infection in age of 6-9 years. Even though both populations live in almost the same place in Singapore, the NPC incidence is high in Chinese population, until the second generation who live in other non-endemic areas. Genetics is the primary factor for the emergence of NPC, supported by cytogenetic findings in NPC tumor tissue, abnormalities in chromosomes 1, 3, 11, 12, 17, and correlation with certain HLA system of NPC patients. Several studies indicate that in the process on NPC development, environmental influence, such as culture/habit and diet, serves as co-factors, in addition the genetic factor (Muir et al. 1987).

Previous studies disclose a strong correlation between NPC patients with history of nasal disorders, the use of Chinese traditional medicine, the use of mosquito coil, and nasal rubbing oil. The presence of ester phorbol compound may activate EBV virus or trigger virus proliferation within respiratory epithelial cells. In Malaysia, Hongkong, and Guangxi province (China) salted fish consumption since childhood has a potential to trigger NPC development. The presence of nitrosamine and its presubstance (NDMA=N-nitrosodimethylamine) may stimulate DNA mutation in nasopharyngeal tissue (Ho 1972; Zheng et al. 1994).

This study used nested RT-PCR with target mRNA Ck 19 (cytokeratin 19) to detect the presence of tumor cells ($1 : 10^7$ normal cells) in peripheral blood circulation of NPC patients (Bustin et al. 1999; Dingemans et al. 1997). Cytokeratin 19 is a filament protein between those expressed by all malignant epithelial cells and and not expressed by lymphoid cells or hematopoietic mesenchymal cells, so that Ck 19 can be used as an early marker of metastasis into peripheral blood, bone marrow, and lymph nodes in patients with Ca originates from epithelial cells. Several other tumor markers, such as PSA (Prostate Specific Antigen) in prostate Ca, CEA (Carcino Embryonic Antigen) in colon Ca, pulmonary Ca, and breast Ca, AFP (Alpha Fetal Protein) in Hepatoma and SCC (Squamous Cell Carcinoma Antigen) in Cervical Ca, can be used as target genes to detect the presence of micrometastasis based on PCR examination. However, NPC does not have tumor specific gene as early tumor marker.

Therefore, this study employed Ck 19, which was expressed by malignant nasopharyngeal epithelial cells, as target molecule and the early marker of micrometastasis in NPC patients for optimizing treatment results and improve the prognosis of NPC

patients. By the finding of early molecular marker (ck 19 mRNA) that indicates the presence of NPC tumor cell micrometastasis, patient management can be improved, such as by the early provision of combined therapy, i.e., radiation and chemotherapy, that can only be undertaken by improving diagnostic accuracy and tumor staging, which, at the same time, also determining the type of treatment and the prognosis of NPC patients.

MATERIALS AND METHODS

Subjects comprised 30 NPC patients in various stages of disease (the criteria of Union International Centre of Cancer 1986 and WHO 1987) and 10 non-NPC ENT patients (normal control) who visited ENT Clinic, Dr Soetomo Hospital, Surabaya. Ten ml venous blood was taken from both groups for RT-PCR. Ethical background of each subject groups was recorded and informed consent as well as ethical eligibility was obtained from Ethical Clearance committee. NPC patients were all patients visiting ENT Clinic, Dr Soetomo Hospital, aged 20-26 years, and no other tumors than NPC. Those classified as normal were patients visiting ENT clinic, Dr Soetomo Hospital, aged 20-60 years, healthy, and had no tumor in other issues.

Venous blood 10 ml (in 15 ml centrifugal tube) was centrifugated 1500 ml for 10 minutes and separated from the plasma. Blood cells were added with 40-45 ml buffer solution (155 mM/L NH₄Cl, 7.5 mM/L KHCO₃, 2.5 mM/L K₂CO₃, 0.1 mM/L EDTA; pH = 8) to lysis the erythrocyte. It was washed and centrifugated several times until cell precipitation in white colors were obtained (Jin et al. 2000). In cell pellet (from centrifugation), Trizol LS (Invitrogen, Cat: 10296-010)-chloroform reagent was added to extract total cellular RNA, including Ck19 mRNA. Supernatant layer was removed and mRNA was precipitated by adding isopropyl alcohol solution, and it was subsequently vortexed and centrifugated. mRNA precipitation at the bottom of the tube was washed with 75% ethanol. mRNA was diluted with DEPC water (Jin et al. 2000).

Total cellular mRNA (template RNA 0.01-1 ug) was added with RT PCR mixture (Invitrogen, Cat 12574-026), 60 mol primer (Ck19-1 and Ck 19-2); and 2 x RT-PCR buffer containing 0,4 mM dNTP dan 3,2 mM MgSO₄. The external primer used in this study matched with conserved area of Ck 19 genome, part of exon 3 and exon 6 fragments, which signaled cytokeratin 19 (Ck 19) protein. Ck 19-1= 5'-ACC-ATG-AGG-AGG-AAA-TCA-GTA-C-3' (22 mer). Ck 19-2= 5'-ATC-TTC-CTG-TCC-CTC-GAG-CA-3' (20 mer). RT procedure for synthesizing cDNA was as follows:

incubation in 55 degree C for 30 minutes, followed with hot-start in 94 degree C for 2 minutes, and PCR in 94 degree C for 0.5 minute; 60 degree C for 0.5 minute, and 68 degree C for 1 minute, in 40 cycles, which resulted in DNA fragment of 518 bp, and directly followed with the second PCR with internal primer matched with exon 4 and exon 6 of Ck 19 as follows: Ck 19-3= 5'-CCA-AGA-TCC-TGA-GTG-ACA-TGC-GAA-G-3' (25 mer). Ck 19-4= 5'-GAT-GTC-CAT-GAG-CCG-CTG-GTA-C-3' (22 mer). PCR in the same condition as that in the first PCR resulted in 371 bp fragment, which was analyzed with electrophoresis in 2% agarose with ethidium bromide staining under UV light (Jin et al. 2000).

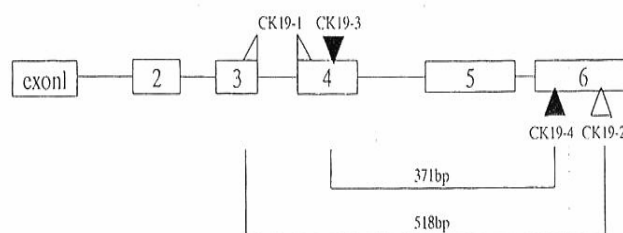


Figure 1. Ck-19 gene structure and relative positions of outer primers (Ck9-1 and Ck9-2 indicated by Δ) and inner primers (Ck19-3 and Ck19-4 indicated by $\blacktriangle$).

RESULTS

This study had involved 30 patients with nasopharyngeal carcinoma in various stages (according to the criteria of Union Internationale Centre of Cancer, 1986 and WHO, 1987) and 10 normal (control) patients with age range of 20-60 years, all were Javanese. From these patients venous blood samples were collected for molecular analysis of Ck 19 mRNA. Molecular analysis of Ck19 mRNA was used to detect the presence of tumor cell micrometastasis in peripheral blood of NPC patients in correlation with their various NPC stages. In Table 1, from 30 NPC patients with age range of 20-60 years, 8 were female and 22 were male. From 10 control patients, 2 were female and 10 were male. All of NPC patients and control were Javanese. Ck19 mRNA was found in the blood of 22 NPC patients (22/30=73.3%), while there was no Ck 19 mRNA (0/10=0%) in control group. The difference was significant ($p < 0.01$). Chi-square estimation revealed $X^2 = 19.39$, which was highly significant for $p < 0.01$.

In Table 2, from 30 NPC patients with various stages according to the criteria of Union Internationale Centre of Cancer (1986) those with stage I was none, stage IIa

1 patient, stage IIb 1 patient, stage III 5 patients, stage IVa 7 patients, and stage IVb 16 patients.

Table 1. Nasopharyngeal carcinoma and Ck 19 mRNA in peripheral blood of NPC patients

NPC	RT-PCR Ck19 peripheral blood		Total
	Positive	Negative	
Positive	22	8	30
Negative	0	10	10
Total	22	18	40

Table 2. Stages of nasopharyngeal carcinoma and Ck 19 mRNA in NPC patients peripheral blood.

NPC stages	RT-PCR mRNA Ck19 in peripheral blood		
	Positive	Negative	Total
Stage I	0	0	0
Stage IIa	0	1	1
Stage IIb	0	1	1
Stage III	3	2	5
Stage IVa	5	2	7
Stage IVb	14	2	16
Total	22	8	30

Analysis of variance revealed $T = 5.103$ (highly significant for $t < 0.02$) and had strong correlation ($R = 0.95$).

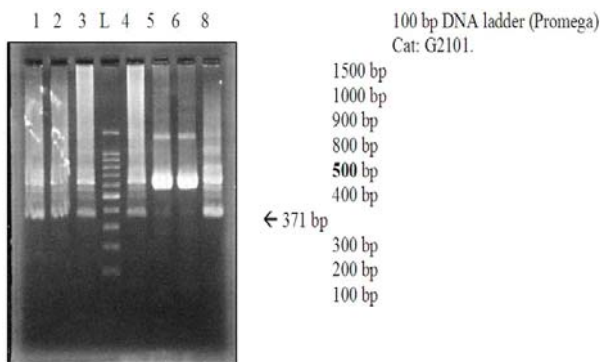


Figure 2. Result of RT-nested PCR of Ck19 mRNA in peripheral blood with Ck19-3 and Ck19-4, producing 371 bp fragment (sample no. 1, 2, 3, 4, and 8). L = 100 bp DNA ladder

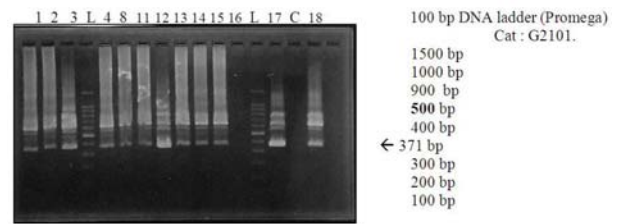


Figure 3. Result of RT-nested PCR of Ck19 mRNA in peripheral blood with Ck19-3 and Ck19-4, producing 371 bp fragment (sample no. 1, 2, 3, 4, 8, 11, 12, 13, 14, 15, 17 and 18). C = negative control L= 100 bp DNA ladder

DISCUSSION

The results of histopathological examination revealed that from NPC patient group, 29 patients (97%) were diagnosed with undifferentiated NPC (WHO type 3) and 1 (3%) was diagnosed with non keratinizing NPC (WHO type 2). This confirmed previous study that reported histopathological results of NPC patients as follows: keratinizing squamous cell NPC (WHO type 1) in 10%, Non keratinizing NPC (WHO type 2) in 5% and undifferentiated NPC (WHO type 3) in 85% (Hu et al. 1996). Walter et al. (1992) in 18 NPC patients found undifferentiated NPC in 78% , keratinizing squamous cell NPC in 11%, and non keratinizing NPC in 11%. The finding of Ck19 mRNA in the blood of NPC patients (73.3%) and the absence of Ck19 mRNA in control group (significantly different $p < 0.01$) indicated that Ck19 mRNA parameter was highly specific and only found in NPC patients with micrometastasis in their blood.

Until recently, Ca diagnosis dy detecting specific gene sequence for a particular Ca type using PCR remains a more sensitive technique compared to other conventional clinical diagnosis, such as X-ray, sonography, computer tomography, magnetic resonance imaging (MRI), and nuclear medical scanning. In this study, we used nested RT-PCR to detect the presence of Ck19 mRNA that was expressed only by NPC epithelial tumor cells, not by lymphoid or other hematopoietic cells, in the peripheral blood of the patients. The sensitivity of RT-PCR technique is very high, approximately $1/10^7$, in which one tumor cell can be detected between 10^7 normal blood cells. To improve the specificity of PCR results, nested PCR using two types of primer, one set outer primer and one set inner primer, was designed. The absence of Ck19 mRNA in all of control group indicated that this parameter had high sensitivity.

Ck19 mRNA was present in 73% of all NPC groups, with details as follows: in stage IIa and IIb, the Ck19 mRNA was not found (0%), in stage III it was found in 60%, stage IVa in 71%, and stage IVb in 88%. These figure indicate that it was highly possible that micrometastasis had occurred and it strongly correlated with further advance of NPC stage (significantly different, $p < 0.02$ and $R = 0.95$). This finding also confirmed the hypothesis that the more advance the NPC stage, the higher the possibility of micrometastasis in NPC patients' blood (hematogenic).

Previous experiences proved that post-radiation therapy failure was due particularly to high recurrence rate of local tumor and tumor metastasis to other tissues. Due to the advance in oncological radiation therapy, for example, by the presence of mega-voltage radiation technique, immobilization improvement, more accurate mapping of tumor expansion localization in computerized photo, and tomographic scan, failure of radiation therapy for NPC patients can be reduced. Up to the moment, therapy failure mainly results from undetected tumor cell metastasis. Until recently, NPC therapy for distant metastasis is far for satisfactory, with 2 year survival of less than 10%. Median survival was only 6 months in untreated patients and 12 months in those receiving chemotherapy only. Therefore, early detection of the presence of subclinical metastasis (micrometastasis) followed with the improvement of therapy quality for NPC patients are two important concerns. From 30 NPC patients in various stages, Ck19 mRNA was found in peripheral blood of 22 patients (73%), so that it was highly possible that micrometastasis and/or distant metastasis had occurred, so that earlier provision of adjuvant and chemotherapy to eradicate micrometastasis may increase the healing rate of NPC patients. It is time to develop RT-PCR quantitative examination of Ck 19 mRNA (real time PCR) to evaluate correlation between Ck19 mRNA level in blood and the patients' clinical prognosis (Stathopoulou et al. 2002; Stathopoulou et al. 2003).

CONCLUSION

The presence of Ck19 mRNA in peripheral blood of nasopharyngeal carcinoma patients is the early marker of micrometastasis. Increased NPC stage is strongly correlated with Ck19 mRNA parameter, indicating a possibility of micrometastasis. Early detection of tumor cell micrometastasis may optimize the result of NPC treatment and prevent recurrence (relapse), so that patient management will be improved. Ck19 mRNA is a specific and sensitive parameter for NPC. RT-PCR quantitative examination should be developed to predict

more accurately the prognosis of NPC patients with suspected metastasis.

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