Diagnostic Usefulness of Telomerase Activity in Nasopharyngeal Carcinoma

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Telomeres are specialized structures at the ends of eukaryotic chromosomes which are composed of simple repetitive G-rich hexameric sequences. Activation of telomerase, a ribonucleoprotein that synthesizes telomeric DNA, is found in most malignant tumors. However, little data is available concerning the correlation between telomerase activity and NPC (nasopharyngeal carcinoma). In this study, telomerase activation was determined using the TRAP (telomerase repeat amplification protocol) assay in 62 nasopharyngeal biopsies (25 NPC, 25 non-malignant nasopharyngeal lymphoid tissues, 12 post-irradiated nasopharyngeal tissues). The results showed that strong telomerase activity was present in both NPC and non-malignant nasopharyngeal biopsies. Post-irradiated nasopharyngeal samples had a significantly lower telomerase activity than NPC and non-malignant nasopharyngeal lymphoid tissues. It is well known that nasopharyngeal tissue is infiltrated by numerous lymphocytes, which might retain telomerase activity. Therefore, the finding that the telomerase activation was lowest in post-irradiated nasopharyngeal tissues is reasonable because of the destruction of activated lymphocytes and NPC by radiation. NPC biopsies with positive lymph node involvement exhibited higher levels of telomerase compared to those without lymph node involvement. Our data indicate a positive association between telomerase activity and tumor potential for lymphatic spreading in limited local tumors. In addition, telomerase activity may be useful as a diagnostic marker in the detection of tumor cells in recurrent NPC, but not in primary NPC.

Key words:    Telomerase activity — Nasopharyngeal neoplasms — Recurrence

Telomeres are specialized structures at the ends of chromosomes that range in size from a few hundred base pairs to approximately 30 kbp.1) Possible functions of telomeres include prevention of chromosome degradation, end-to-end fusion, rearrangements and chromosome loss.2) During the normal aging process of human differentiated cells, a reduction in average telomere length occurs. Telomerase is a ribonucleoprotein that maintains telomere length and whose activity is associated with cellular immortality.3) Telomerase activity has been detected in germline, immortalized and malignant cells.4) Recently, telomerase activity has been detected in the tissues of many human cancers but not in the majority of normal tissues, suggesting that telomere stabilization by telomerase may play a role in tumorigenesis.6, 7) This striking observation led to the suggestion that telomerase might be important for the continuous growth or progression of cancer cells.

High levels of telomerase activity have been found to be associated with an advanced stage and a poor prognosis in leukemia,8) neuroblastoma9) and gastric cancer.10) In contrast to many other human cancers, the significance of telomerase activity in relation to early diagnosis, staging and prognosis of nasopharyngeal carcinoma (NPC) has not been reported. NPC is derived from the nasopharyngeal surface epithelium, particularly in Rosenmüller’s surface. The prevalence rate is low in most of the world (0.5 to 2/100 000 per year), but is high in southern China and Taiwan (25 to 50/100 000 per year). The NPC rate increases slowly after the age of 20 and decreases after 60 in China. The mean age of occurrence is 40 to 50.11, 12) Because the nasopharynx is in a deep anatomical region, NPC is often discovered only when it is locally advanced or has already spread to the regional lymph nodes. NPC diagnosed at an early stage is associated with a better prognosis. Since advances in detection methods have resulted in a polymerase chain reaction (PCR)-based assay that can detect as few as 50 telomerase-expressing cells per sample,9) the enzyme is considered to be an early diagnostic marker of malignancy.13) Such a marker would also be useful in the detection of cancer cells in residual tumor after radiation therapy.

In normal nasopharynx, the pharyngeal mucosa is infiltrated by diffuse lymphoid tissue and organized lymphoid nodules, which belong to Waldenström’s lymphatic ring.14) The area of NPC is also surrounded by intense lymphocytic infiltration whose prognostic relevance is still doubtful.15) Telomerase activity has been detected at low levels

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in peripheral blood lymphocytes and higher levels occur in activated B and T cells.\(^{10}\) In the present study, the telomerase activity in a series of NPC, non-malignant and post-irradiated nasopharyngeal tissues was measured using the telomeric repeat amplification protocol (TRAP) assay. The aim was to evaluate the telomerase activity in nasopharyngeal tissues and carcinoma before and after radiotherapy.

**MATERIALS AND METHODS**

**Patients** The study population consisted of 25 NPC patients (19 males, 6 females) with a mean age of 50.6 years (range, 31–84 years). The non-malignant group included 19 patients with a nasopharyngeal mass which was later confirmed to be negative for NPC and 6 patients with other head and neck cancer (17 males and 8 females) with a mean age of 44.9 years (range, 15–76 years). There were 12 patients (5 males, 7 females) who received radiation therapy due to NPC, with a mean age of 51.2 years (range, 36–70 years).

**Tissue samples** Sixty-two nasopharyngeal biopsies (25 tumor samples, 25 non-malignant and 12 post irradiated nasopharyngeal tissue samples) were obtained for analysis. Informed consent for the use of clinical materials in telomerase study was obtained from all patients. The fresh blocks were cut into 2 pieces, of which one was used for pathologic examination and the other was stored at −70°C for later use. The nasopharyngeal biopsies were examined by cryosectioning to reconfirm the presence or absence of neoplastic cells before TRAP assay.

**Histology** The histological sections of NPC cases were examined and grouped according to the principal histological classification of the World Health Organization (WHO), taking into account the degree of lymphoid infiltration. They subdivided the WHO types II and III in the subtypes a) without and b) with lymphoid infiltration. In the non-malignant group, major lymphoid infiltration was defined as 50 and more lymphocytes per viewing field at 10×25 (ocular×objective) magnification. In this report we used the term ‘lymphoid hyperplasia’ for the lymphoid infiltration with germinal centers.

**Clinical staging** The clinical staging of the nasopharyngeal carcinoma was performed according to the TNM staging system (International Union Against Cancer [UICC] 1997). All patients received complete head and neck local examinations. Imaging studies (bone scans, abdominal ultrasonography, computed tomography [CT] or magnetic resonance imaging [MRI]) were applied to confirm the clinical staging.

**TRAP assay** TRAP assay was performed as described previously,\(^4,17\) with minor modification. Briefly, ten sections of 10 µm frozen-section tissues were washed in ice-cold phosphate-buffered saline (GIBCO BRL, Gaithersburg, MD), pelleted, and homogenized with 100 µl of 1× CHAPS lysis buffer [10 mM Tris-HCl (pH 7.5), 1 mM MgCl\(_2\), 1 mM ethylene glycol-bis[β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid (EGTA), 0.1 mM benzamidine, 5 mM β-mercaptoethanol, 0.5% 3-[3-cholamidopropyl]dime thylamino]-1-propanesulfonate (CHAPS), and 10% glycerol] in Kontes tubes with matching pestles rotated at 600 rpm. After 30 min on ice, the lysate was centrifuged at 16000\(g\) for 20 min at 4°C. The supernatant was poured into microtubes, quickly frozen in dry ice and stored at −70°C. The protein concentration of the supernatant was determined with the bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL). The TRAP assay was performed in a 12.5 µl reaction mixture using the TRAPEze telomerase detection kit (Oncor Inc., Gaithersburg, MD) according to the manufacturer’s protocol, with some modifications. Briefly, 1 µg of forward primer (FP) was labeled using 2.5 units T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and 25 µCi of 3000 Ci/mmol [γ-\(^{32}\)P] adenosine triphosphate (Amersham International plc, Buckinghamshire, UK). Each TRAP reaction mixture consisted of 1.25 µl of 10× TRAP buffer, 50 µM dNTPs, 25 ng end-labeled FP, 25 ng reverse primer, 25 ng of internal control primer, 0.025 amol of the 36-bp internal control template, 0.25 units of TaKaRa Taq DNA polymerase (TaKaRa Shuzo Co., Ltd., Shiga), and 0.5 µg of protein extract. After 30-min incubation at 30°C for telomerase-mediated extension of the FP, the reaction mixture was heated at 95°C for 2 min and then immediately subjected to 30 PCR cycles of 95°C for 50% and 58°C for 30 s each. The PCR products were electrophoresed on an 8% nonde natured polyacrylamide gel, which was then autoradiographed. Any sample that showed no 36-bp internal control primer PCR was considered a false-negative sample containing DNA polymerase inhibitors. Telomerase activity was considered to be positive when a 6-bp ladder was observed after overnight exposure at −70°C (Fig. 1).

For confirmation of TRAP results by autoradiography as described above, another semi-quantitative telomerase assay was also performed using a telomerase PCR-ELISA kit (Boehringer Mannheim, Mannheim, Germany) based on the TRAP method.\(^{18}\) The supernatant containing 5 µg protein was used for PCR. The thermal cycling was carried out under the following conditions: primer elongation for 20 min at 25°C, telomerase inactivation for 5 min at 94°C, product amplification by repeat of 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 50°C) and polymerization (90 s at 72°C). Telomerase activity was detected as a color change of 3,3′,5,5′-tetrathymethyl benzidine with peroxidase and was expressed as an absorbance. Samples were regarded as telomerase-positive if the difference in absorbance was higher than 0.2 \(A_{490\text{nm}}/A_{590\text{nm}}\). According to the manufacturer’s manual, the maximum value of absorbance is 3.9. Data over 3.9 were treated as 3.9.
Controls were included in each assay: positive control (HeLa cells), negative control 1 containing no protein, and negative control 2 predigestion of protein extracts with 1 µg/µl DNase-free RNase (Boehringer Mannheim) at 37°C for 30 min. RNase treatment was only performed in selected cases because it is easy to distinguish a truly telomerase-positive extract from an artifact.

Statistical analysis Statistical analysis was performed using Fisher’s exact test and the Mann-Whitney U test to evaluate the significance of differences between NPC, non-malignant and post-irradiated nasopharyngeal tissues. A value of $P<0.05$ was considered statistically significant.

RESULTS

Telomerase activity was present in 21 out of 25 NPC specimens (84%), in 21 out of 25 non-malignant nasopharyngeal lymphoid tissues (84%), and in 4 out of 12 post-irradiated nasopharyngeal tissues (33%). No difference of telomerase expression was observed between biopsies.

Table I. Telomerase Activity in 25 Patients with Nasopharyngeal Carcinoma

| Case No. | Sex | Age | TNM    | Clinical staging | Histological type | Telomerase activation<sup>a</sup> | ELISA (absorbance)<sup>b</sup> |
|----------|-----|-----|--------|------------------|-------------------|----------------------------------|---------------------------------|
| NPC1     | F   | 57  | T2bN2M0| III              | IIa               | +                                | 1.053                           |
| NPC2     | F   | 48  | T2bN2M0| III              | IIa               | +                                | 2.288                           |
| NPC3     | M   | 65  | T1N1M0 | IIb              | IIb               | +                                | 1.957                           |
| NPC4     | F   | 45  | T4N1M0 | IVA              | IIb               | +                                | 3.404                           |
| NPC5     | M   | 35  | T1N1M0 | IIb              | IIb               | +                                | 1.452                           |
| NPC6     | M   | 63  | T2bN0M0| IIb              | I                 | +                                | 0.627                           |
| NPC7     | F   | 49  | T3N2M0 | III              | IIb               | +                                | 0.404                           |
| NPC8     | M   | 42  | T1N1M0 | IIb              | IIb               | +                                | 3.900                           |
| NPC9     | M   | 63  | T1N2M0 | III              | IIb               | +                                | 3.344                           |
| NPC10    | M   | 53  | T1N3bM0| IIV              | IIIa              | +                                | 2.571                           |
| NPC11    | M   | 60  | T2bN2M0| III              | IIb               | +                                | 3.900                           |
| NPC12    | M   | 46  | T2aN0M0| IIa              | IIb               | −                                | 0.157                           |
| NPC13    | M   | 44  | T1N1M0 | IIb              | IIb               | +                                | 0.682                           |
| NPC14    | M   | 57  | T4N1M0 | IVA              | IIb               | +                                | 0.292                           |
| NPC15    | F   | 84  | T2bN0M0| IIb              | IIb               | −                                | 0.194                           |
| NPC16    | M   | 45  | T2bN2M0| III              | IIb               | +                                | 0.249                           |
| NPC17    | M   | 49  | T1N0M0 | I                | IIa               | +                                | 2.039                           |
| NPC18    | M   | 43  | T4N1M0 | IVA              | IIb               | +                                | 1.591                           |
| NPC19    | M   | 40  | T2N1M0 | IVA              | IIb               | −                                | 0.055                           |
| NPC20    | F   | 73  | T4N0M0 | IVA              | IIIa              | +                                | 0.066                           |
| NPC21    | M   | 42  | T2N1M0 | IIb              | IIb               | +                                | 3.900                           |
| NPC22    | M   | 47  | T4N2M0 | IVA              | IIa               | +                                | 1.375                           |
| NPC23    | M   | 36  | T1N0M0 | I                | IIb               | −                                | 0.039                           |
| NPC24    | M   | 47  | Recurrence |              |                   | +                                | 2.864                           |
| NPC25    | M   | 31  | Recurrence |              |                   | +                                | 2.985                           |

<sup>a</sup> Evaluation of telomerase activity was done by autoradiography of the TRAP product.
<sup>b</sup> A value of absorbance over 3.9 was treated as 3.9.
from NPC and nasopharyngeal lymphoid tissues ($P>0.05$; Fisher’s exact test). However, the frequency of telomerase activation was significantly higher in both the NPC and non-malignant nasopharyngeal lymphoid tissues compared to that in the post-irradiated nasopharyngeal tissues ($P=0.004$).

Telomerase activation was common in all stages of NPC: 50% in stage I (one out of 2); 75% in stage II (6 out of 8); 100% in stage III (6 out of 6) and 83% in stage IV (6 out of 7). No statistically significant difference in the degree of telomerase activation was observed between specimens from the early stages (stage I and II) and advanced stages (stage III and IV) of NPC (Table I). However, telomerase activation in NPC specimens with positive lymph node involvement was significantly higher (16 out of 17, 94%) than in specimens with negative lymph node involvement (3 out of 6, 50%) ($P=0.04$).

Semi-quantitative telomerase activity in NPC specimens (1.66±1.38 absorbance) and that in non-malignant nasopharyngeal lymphoid tissues (2.46±1.36 absorbance) were not significantly different (Mann-Whitney $U$ test) (Fig. 2). In contrast, telomerase activity in post-irradiated specimens (0.48±0.97 absorbance) was significantly lower than in the neoplastic group ($P=0.032$) and the non-malignant group ($P=0.012$). Telomerase activity in patients with lymph node metastases (1.91±1.38 absorbance) and with tumor-free lymph nodes (0.52±0.77 absorbance) were significantly different ($P=0.012$).

![Fig. 2. (A) Telomerase activity in nasopharyngeal carcinoma (C), non-malignant (N) and post-irradiated nasopharyngeal biopsies (P). (B) Telomerase activity in NPC from patients with negative (N−) and with positive (N+) lymph nodes. The number of patients is given under each bar.](image)

![Fig. 3. Nasopharyngeal subepithelium is infiltrated by major lymphoid tissue and organized germinal centers. (×40)](image)

| Case No. | Sex | Age | Time after irradiation | Histological findings | Telomerase activationa) | ELISA (absorbance) |
|----------|-----|-----|------------------------|-----------------------|-------------------------|-------------------|
| RT1      | M   | 70  | 4 years                | Granulation           | –                       | 0.058             |
| RT2      | M   | 40  | 5 months               | Fibrosis              | –                       | 0.107             |
| RT3      | M   | 57  | 14 years               | Epithelial tissue     | +                       | 0.367             |
| RT4      | F   | 57  | 4 months               | Fibroleucocyte        | –                       | 0.099             |
| RT5      | M   | 57  | 19 months              | Fibroleucocyte        | +                       | 1.210             |
| RT6      | F   | 45  | 26 months              | Necrosis              | –                       | 0.037             |
| RT7      | M   | 43  | 1 month                | Necrosis              | –                       | 0.108             |
| RT8      | F   | 36  | 28 months              | LHb)                 | +                       | 3.389             |
| RT9      | F   | 41  | 19 months              | Epithelial tissue     | –                       | 0.051             |
| RT10     | F   | 42  | 11 years               | Granulation           | +                       | 0.290             |
| RT11     | F   | 68  | 10 years               | Granulatton           | –                       | 0.025             |
| RT12     | F   | 58  | 9 months               | Necrosis              | –                       | 0.027             |

a) Evaluation of telomerase activity was done by autoradiography of the TRAP product.
b) LH: lymphoid hyperplasia (lymphoid infiltration with germinal centers).
The results of histologic classification of NPC are given in Table I. No obvious relationship with telomerase activity was observed between WHO type I, II and III cases. Lymphoid infiltration was a common characteristic in the type II and III cases. No difference of telomerase expression was observed between biopsies from NPC with (12/16) and without (6/6) lymphoid infiltration (P>0.05; Fisher’s exact test). In the non-malignant nasopharyngeal lymphoid tissues, major lymphoid infiltration was found in 72% (18/25) of the patients and lymphoid hyperplasia was diagnosed in 64% (16/25) of the patients (Fig. 3). No statistically significant difference in the degree of telomerase activation was observed between specimens with major (16 out of 18, 88.9%) and minor (5 out of 7, 71.4%) lymphoid infiltration. However, telomerase activation in non-malignant specimens with lymphoid hyperplasia was significantly higher (16 out of 16, 100%) than in specimens without lymphoid hyperplasia (5 out of 9, 55.6%) (P=0.01).

In the post-irradiated group, the time of sample collection ranged between 1 month and 14 years after radiation therapy (Table II). Lymphocyte depletion is the most commonly observed pattern in the post-irradiated nasopharyngeal tissues (Fig. 4). In one of our 4 telomerase-positive post-irradiated cases, a negative biopsy result showed granulation with bizarre spindle cells. In case 10, because a rapid progressive nasopharyngeal mass with skull base invasion was found on MRI within 2 months, treatment as a recurrent NPC was given despite the lack of pathological proof. One case with lymphoid hyperplasia in post-irradiated nasopharynx also expressed telomerase activity. Although the pathological and imaging studies showed negative findings for malignancy or lymphoid hyperplasia in the other 2 telomerase-positive cases, the patients were closely followed up for the possibility of tumor recurrence.

DISCUSSION

The result of the study revealed that telomerase activity is frequently expressed in non-malignant nasopharyngeal lymphoid tissues. The findings of a previous study revealed that the subepithelial layer of nasopharynx contains processes of nodular lymphoid tissue (about 2 mm thick). This is presumably why a nasopharyngeal biopsy without including subepithelial lymphoid tissue is almost impossible. The nasopharyngeal lymphoid tissue and palatine tonsil all belong to Waldeyer’s lymphatic ring, which is putatively the first line of immunologic defense mechanisms. Different stimulation mechanisms for the activation of telomerase are likely to exist, one of these being immune stimulation. Human palatine tonsil was found to express telomerase activity that was as high as that in lymphoma. This strong telomerase activity seemed to be derived from normal germinal centers, which were also commonly found in activated nasopharyngeal lymphoid tissues. It is thus reasonable that non-malignant nasopharyngeal tissue presenting an inflammatory focus, as well as many activated lymphoid cells, also expresses telomerase activity.

Many studies in human malignancies have revealed a relation between the telomerase activity and some known prognostic factors, such as lymph node status, tumor size and clinical staging. However, other studies have failed to confirm these results and found no correlation between telomerase activity and clinical outcome or other known prognostic indicators. Our data support the finding of Cheng et al. that telomerase activity was significantly lower in NPC without lymph node involvement than in NPC with positive lymph node metastases. There is a positive association of telomerase activity with tumor potential for lymphatic spreading in limited local tumors. In clinical staging, a higher frequency of telomerase activation was observed in the advanced stage of NPC (92%) compared with the early stage of the disease (70%). However, this difference was not statistically significant. No correlation between histological classification or size of tumor and telomerase activity was observed.

The present study is the first to report telomerase activity in post-irradiated nasopharyngeal tissues. The extreme radiosensitivity of lymphocytes is a common radiobiological characteristic of most lymphoid tissues. Lymphocyte depletion is the most commonly observed pattern in post-irradiated nasopharyngeal tissues. In this study, a significantly lower frequency of telomerase activation was observed in irradiated tissues compared with NPC and non-malignant biopsies. A probable explanation for this phenomenon is that destruction of cancer and nasopharyngeal tissues was a common characteristic in the type II and III cases. No difference of telomerase expression was observed between biopsies from NPC with (12/16) and without (6/6) lymphoid infiltration (P>0.05; Fisher’s exact test). In the non-malignant nasopharyngeal lymphoid tissues, major lymphoid infiltration was found in 72% (18/25) of the patients and lymphoid hyperplasia was diagnosed in 64% (16/25) of the patients (Fig. 3). No statistically significant difference in the degree of telomerase activation was observed between specimens with major (16 out of 18, 88.9%) and minor (5 out of 7, 71.4%) lymphoid infiltration. However, telomerase activation in non-malignant specimens with lymphoid hyperplasia was significantly higher (16 out of 16, 100%) than in specimens without lymphoid hyperplasia (5 out of 9, 55.6%) (P=0.01).

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geal lymphoid cells by previous radiation therapy also abolished the telomerase activity of these cells. Early and accurate detection of recurrence in previously treated NPC remains a major diagnostic challenge. Negative biopsy results including granulation tissue do not necessarily exclude tumor recurrence, as was demonstrated in one of our 4 telomerase-positive post-irradiated cases. Without the influence of lymphoid tissues, telomerase activation has potential as a marker to improve early detection of tumor recurrence in the post-irradiated nasopharyngeal biopsies.

There is a problem when we measure telomerase activity in NPC biopsies. Because NPC is characterized by a remarkable infiltration of lymphocytes in the tumor tissue,\(^2\) the telomerase activity in NPC specimens may be derived from cancer cells or benign lymphocytes. The significant telomerase activity in benign lymphoid tissues seemed to be mainly expressed in germinal center B cells.\(^3\) A low level of telomerase activity was expressed in resting mature lymphocytes. Germinal centers were seldom found in NPC specimens, because tumor-infiltrating lymphocytes in NPC were mainly mature T lymphocytes.\(^2\)\(^7\)\(^8\) Telomerase activity correlated with B-cell numbers and cell proliferation in benign lymphoid tissues. If germinal centers were absent in NPC specimens, the influence of tumor-infiltrating lymphocytes in telomerase activity might be minimal.

From a clinical point of view, the usefulness of telomerase activity as a diagnostic marker and in predicting prognosis remains the subject of debate. However, our results suggest that the use of telomerase activation in the early detection of occult primary NPC is almost impossible because of the interference of high telomerase activity in activated nasopharyngeal lymphoid tissue. Nevertheless, telomerase activity may serve as a potential marker in the detection of recurrent tumor cells because nasopharyngeal lymphoid tissue is usually destroyed by previous radiation therapy. Although a high frequency of telomerase activation is observed in cervical metastatic NPC, determination of the prognostic value of the level of the telomerase activation in NPC will require further studies involving a long period of follow up.

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