Demonstration of Epstein-Barr Virus-specific DNA Polymerase in Chemically Induced Raji Cells and Its Antibody in Serum from Patients with Nasopharyngeal Carcinoma

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ABSTRACT

Epstein-Barr virus (EBV) has been found to be associated with nasopharyngeal carcinoma (NPC), and antibodies with high frequency and titer to EBV proteins have been found in sera from NPC patients. Raji cells, an EBV genome-carrying nonproducer cell line, treated with 12-O-tetradecanoylphorboI-13-acetate and n-butylate induced a unique EBV DNA polymerase which has properties similar to the EBV DNA polymerase encoded by 12-O-tetradecanoylphorboI-13-acetate in P3HR-1 cells, an EBV producer cell line. The possible presence of antibodies to this EBV DNA polymerase in NPC patient serum was examined. The mean number of EBV DNA polymerase units neutralized was 380 ± 168 units/ml serum (mean ± SD) in 48 sera from patients with NPC, whereas that in the sera from 52 healthy donors was 62 ± 56 units/ml (p < 0.01). The EBV DNA polymerase antibody was found to be associated with the immunoglobulin G but not the immunoglobulin A fraction, and its titer was not correlated with the titers against EBV DNase or virus capsid antigen-immunoglobulin A. Whether the EBV DNA polymerase antibody is against the EBV DNA polymerase core protein or its stimulating protein is still being investigated. This study demonstrated the high frequency and high titer of antibody against EBV DNA polymerase in serum from NPC patients and suggested the potential of utilizing this antibody titer to complement other methods for the early diagnosis or prognosis of NPC.

INTRODUCTION

EBV3 has been found to have a close association with Burkitt's lymphoma and NPC (1, 2). The DNA of this herpes virus could be responsible for the synthesis of several virus-specific proteins in EBV producer cell lines such as P3HR-1. Among them, several activities, such as DNA polymerase, its stimulating protein, and DNase have been demonstrated (3-5). Biopsies of NPC and Burkitt's lymphoma usually reveal the presence of EBV DNA and EBV nuclear antigen (6-10). Recently, activities similar to EBV DNase and EBV DNA polymerase from P3HR-1 cells were observed in crude extracts of NPC biopsies and in the metastatic lymph node from NPC patients (11, 12). The EBV DNA polymerase in metastatic lymph nodes from NPC patients has been partially purified and characterized. The enzyme characteristics were found to be similar to that from P3HR-1 cells (13). A high frequency and high level of antibody to EBV DNase were observed in sera from patients with NPC (14, 15). A correlation between the prognosis of NPC patients and the serum titer of antibody to EBV DNase was reported (16). Since both EBV DNA polymerase and DNase were found to be present in tumor biopsies from NPC patients, it would be of interest to examine the possible presence of antibody to EBV DNA polymerase in the serum from patients with NPC.

One major difficulty in studying EBV DNA polymerase and DNase is the source of enzyme. In our previous studies these enzymes were studied using EBV carrying cells. Specifically, P3HR-1 cells were exposed to TPA; the induction process for expression of these enzyme activities is tedious. Recently, it was reported that EBV DNA polymerase and DNase might also be induced in Raji cells after combined treatment with TPA and n-butyrate (17, 18). It was not clear whether the properties of the DNA polymerase induced were the same as those induced in P3HR-1 cells, since those studies used quite crude preparations. In this report, we confirm previous observations in terms of induction of EBV DNA polymerase and DNase in Raji cells after their treatment with TPA and n-butyrate (17, 18). This EBV DNA polymerase-like activity was further purified and compared with that derived from P3HR-1 cells. Furthermore, using EBV DNA polymerase derived from chemically induced Raji cells as an antigen, we have demonstrated the presence of antibody against EBV DNA polymerase with high titer and frequency in serum from patients with NPC. The relationship of this titer to other titers of antibodies against EBV unique antigens, which were also found to be associated with NPC, was also examined.

MATERIALS AND METHODS

Cells. Raji cells were grown at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum and 100 µg/ml of kanamycin. To induce viral enzymes, cells were treated with 30 ng of TPA per ml and 4 mM n-butyrate for 48 h unless stated otherwise.

Enzyme Extraction. After induction with TPA and n-butyrate for 48 h, Raji cells were pelleted by centrifugation, washed with phosphate-buffered saline twice, then resuspended in extraction buffer (50 mM Tris-HCl, pH 7.5, 0.3 M KCl, 5 mM b-mercaptoethanol, 0.7 mM phenylmethylsulfonyl fluoride, and 20% glycerine) at a density of 2.5 x 10^6 cells/ml, and sonicated for 20 s (4 times) with a Branson sonifier. The enzyme extract was centrifuged at 12,000 rpm for 20 min at 4°C, and the supernatant was collected as the crude enzyme extract.

Purification of EBV DNA Polymerase and SP. The procedures used were essentially the same as those previously published from this laboratory (4, 5). It involved DEAE-, phospho-, and DNA-cellulose column chromatography as described.

EBV DNA Polymerase Assay. EBV DNA polymerase activity was measured as described previously, and a unit of enzyme activity is the same as that defined previously (4). Determination of Anti-EBV DNA Activity. This procedure was the same as that described by Cheng et al. for exonuclease (11).

Human Sera. Fifty-two normal human sera samples were obtained from healthy donors and laboratory personnel of whom 20% were of Chinese origin; 48 sera were obtained from patients with NPC. The latter were collected before radiotherapy (from the Cancer Institute, Chinese Academy of Medical Sciences, Beijing, China). The diagnosis of patients with NPC was made by histopathology. Sera with known titers of VCA-IgA and EA(D)-IgA were kindly provided by St. Jude's Hospital, Memphis, TN, for previous anti-EBV DNase studies.

Fractionation of Serum IgG. Sera from 5 patients with NPC that had a strong titer of antibody to EBV DNA polymerase were pooled, and...
Ten μl of enzyme extract (0.6-0.8 units) was incubated with 10 μl of column using differential pH elution according to the procedure reported by Ey et al. (19).

Neutralization of EBV DNA Polymerase Activity by Human Sera. Ten μl of enzyme extract was incubated with 10 μl of serum diluted (1:8 to 1:10) with 20 mM Tris buffer, pH 7.5, for 20 min at 20-25°C. EBV DNA polymerase activity was assayed and the difference between the enzyme activity in the presence and absence of serum was calculated. The titer of antibody to EBV DNA polymerase was expressed as the units of DNA polymerase activity neutralized by 1 ml of undiluted serum.

Immunoprecipitation of EBV DNA Polymerase with NPC Sera. Immunoprecipitation of EBV DNA polymerase was performed by adding 15 μl of diluted (1:5) sera from patients with NPC or from healthy donors with 15 μl of phosphocellulose-purified EBV DNA polymerase (Fig. 1B, peak II), which was separated from SP. After incubation for 20 min at room temperature, 15 μl of preswelled Protein A-Sepharose 4B was added and the mixture was mixed gently. After a second incubation for 1 h at 4°C, the immunocomplexes were pelleted by centrifugation, and a 10-μl aliquot of the supernatant was examined for DNA polymerase activity in the presence or absence of 10 μl of purified SP.

RESULTS

Purification of Salt-Dependent DNA Polymerase Induced in Raji Cells. Raji cells were treated with TPA and n-butyrate for 48 h. The activities of DNase and the salt-dependent DNA polymerase were induced in treated Raji cells, with that of DNA polymerase being more than 80-fold relative to nontreated Raji cells (data not shown). DEAE-, phospho-, and DNA-cellulose columns were used sequentially to purify the induced salt-dependent DNA polymerase. As shown in Fig. 1A, the salt-dependent DNA polymerase was retained on the DEAE-cellulose column and then eluted as a sharp peak at 0.14 M phosphate. No similar peak of salt-dependent DNA polymerase activity was observed from the untreated Raji cells (data not shown). Further purification of DNA polymerase in the DEAE-cellulose eluates (Fig. 1A) was carried out on a phosphocellulose column. As shown in Fig. 1B, EBV DNA polymerase could be absorbed on the column and was eluted at 0.3 M phosphate (Fig. 1B, peak II). As in the case for purification of EBV DNA polymerase from P3HR-1 cells, several fractions (Fig. 1B, peak I) which were eluted ahead of the EBV DNA polymerase activity peak were demonstrated to be able to stimulate EBV DNA polymerase activity (Fig. 1B, peak II). The fractions with EBV DNA polymerase activity from the phosphocellulose column were pooled and purified further on a DNA-cellulose column. As shown in Fig. 1C, the EBV DNA polymerase was eluted at about 0.3 M KCl, and this EBV DNA polymerase preparation had a specific activity of 405 units/mg of protein.

Characterization of Purified Salt-Dependent DNA Polymerase. It was demonstrated that the EBV DNA polymerase activity was salt dependent (Fig. 2A). Ammonium sulfate [(NH₄)₂SO₄] at 50, 100, and 150 mM was able to stimulate the purified DNA polymerase activity. At 100 mM ammonium sulfate, a 6-fold increase in enzyme activity was observed. In the presence of 150 mM KCl, the activity of this enzyme was three times as high as that without KCl. It was also found that this enzyme was sensitive to phosphonoformate; about 48% of the DNA polymerase activity was inhibited in the presence of 5 μM PFA (Fig. 2B). These properties are the same as those previously found for EBV DNA polymerase purified from P3HR-1 cells (3, 4). It strongly suggests that the salt-dependent DNA polymerase demonstrated in the present study is the same as the EBV polymerase from P3HR-1 cells. In this manuscript, we have termed this salt-dependent DNA polymerase as EBV DNA polymerase.

Presence of Antibody to EBV DNA Polymerase in Sera of Patients with NPC. As shown in Fig. 3, EBV DNA polymerase activity in crude extracts of induced Raji cells could be neutralized by pooled sera from patients with NPC. The neutralization followed a dose-response pattern. When the EBV DNA polymerase activities of DEAE- or phosphocellulose purified enzyme preparations were used, a similar dose-response relationship of anti-EBV DNA polymerase activity and NPC serum was observed. The serum from healthy donors examined in a similar fashion showed no neutralization of EBV DNA polymerase activity (data not shown).
and covered 55% of the neutralizing effect of serum IgG; peak VI contained the highest amount of serum IgG. It was found that peak VI contained the highest amount of serum IgG to contain different amounts of IgG subclasses (19, 22). It was found that peak VI contained the highest amount of serum IgG to contain different amounts of IgG subclasses (19, 22). It was found that peak VI contained the highest amount of serum IgG to contain different amounts of IgG subclasses (19, 22). It was found that peak VI contained the highest amount of serum IgG to contain different amounts of IgG subclasses (19, 22). It was found that peak VI contained the highest amount of serum IgG to contain different amounts of IgG subclasses (19, 22).

Polymerase were pooled and the Ig fractions were fractionated as described previously (20, 21). The DNA polymerase of HSV-I and HSV-II was purified from HSV-I (KOS strain) and HSV-II (333 strain)-infected HeLa cells, respectively.

Table 1 Immunospecificity of serum antibody from patients with NPC to EBV DNA polymerase

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>+Normal serum</th>
<th>+NPC serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBV (Raji cells)</td>
<td>0.61</td>
<td>0.07</td>
</tr>
<tr>
<td>EBV (P3HR-1 cells)</td>
<td>0.60</td>
<td>0.06</td>
</tr>
<tr>
<td>HSV-I</td>
<td>0.62</td>
<td>0.56</td>
</tr>
<tr>
<td>HSV-II</td>
<td>0.61</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Immunospecificity of Antibody to EBV DNA Polymerase Activity in Human Serum. In order to know the immunospecificity of antibody to EBV DNA polymerase activity present in NPC serum, HSV-I and HSV-II DNA polymerase purified as reported previously (20, 21) were used for comparison. These data are shown in Table 1. About 90% of the EBV DNA polymerase activity could be neutralized by the NPC serum; only 8% of HSV-I enzyme and 5% of HSV-II enzyme were neutralized by the same NPC serum.

Characterization of Serum Antibody to EBV DNA Polymerase. Sera which had a high titer of antibody to EBV DNA polymerase were pooled and the Ig fractions were fractionated on a Protein A-Sepharose column; the results are presented in Fig. 4. Six immunoglobulin fractions (peaks I–VI) were obtained. Peak I, which did not bind to the column, might contain IgA, IgM, IgG3, and other serum proteins (22); no neutralization effect on EBV DNA polymerase activity was observed in this fraction. Peaks II–VI, which were bound on the column, were fractionated by several stepwise elutions using buffers at different pH values (6.0, 5.0, 4.5, 4.0, and 3.0) and were found to contain different amounts of IgG subclasses (19, 22). It was found that peak VI contained the highest amount of serum IgG and covered 55% of the neutralizing effect of serum IgG; peak V was next and covered 18%, peaks IV, III, and II covered 15, 7.5, and 4.5%, respectively. Thus, the IgG in peaks IV, V, and VI covered more than 85% of the total serum neutralizing effect on EBV DNA polymerase.

We examined the issue of whether antibodies that neutralize EBV DNA polymerase are the same as the antibodies to the SP in NPC serum which we had demonstrated previously (4). In order to do this, we used the DNA-cellulose-purified EBV DNA polymerase preparation (Fig. 1C) that was separated from the SP (Fig. 1B, peak I) as the antigen. The DNA polymerase was mixed with the serum antibody (or antibodies), and then the antigen complexes were absorbed by Protein A-Sepharose in the presence or absence of SP; results are shown in Table 2. When the EBV DNA polymerase was incubated with normal serum and absorbed by Protein A-Sepharose, there was 0.22 units of EBV DNA polymerase activity; after adding adequate amounts of stimulating protein (Fig. 1B, peak I), the EBV DNA polymerase activity rose to 0.35 units. When EBV DNA polymerase was incubated with NPC serum and absorbed by Protein A-Sepharose, the activity dropped to 0.03 units. The EBV DNA polymerase activity which had been absorbed by Protein A-Sepharose could not be restored to 0.35 or 0.22 units even when replenished with adequate amounts of SP. Therefore it appears that the antibodies in NPC serum that can neutralize the EBV DNA enzyme protein are different from those specific for the stimulating protein of EBV DNA polymerase.

Apparent Frequency and Titer of Antibody to EBV DNA Polymerase. Our studies could not distinguish between antibodies against EBV DNA polymerase or its stimulating protein. Thus, we have termed it as an apparent frequency or titer in this study. The data are summarized in Fig. 5. Fifty-two sera from healthy donors were examined and the activity of EBV DNA polymerase was mixed with the serum antibody (or antibodies), and then the antigen complexes were absorbed by Protein A-Sepharose in the presence or absence of SP; results are shown in Table 2. When the EBV DNA polymerase was incubated with normal serum and absorbed by Protein A-Sepharose, there was 0.22 units of EBV DNA polymerase activity; after adding adequate amounts of stimulating protein (Fig. 1B, peak I), the EBV DNA polymerase activity rose to 0.35 units. When EBV DNA polymerase was incubated with NPC serum and absorbed by Protein A-Sepharose, the activity dropped to 0.03 units. The EBV DNA polymerase activity which had been absorbed by Protein A-Sepharose could not be restored to 0.35 or 0.22 units even when replenished with adequate amounts of SP. Therefore it appears that the antibodies in NPC serum that can neutralize the EBV DNA enzyme protein are different from those specific for the stimulating protein of EBV DNA polymerase.
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Fig. 5. Titer of antibody to EBV DNA polymerase in sera from healthy donors and patients with NPC. An aliquot (10 μl) of EBV DNA polymerase (0.6–0.8 units of DEAE-cellulose purified enzyme) was incubated with 10 μl of 1:10 diluted serum at room temperature for 20 min. In control reactions, 10 μl of heat-inactivated bovine serum albumin (6 mg/ml) was added instead of diluted serum. The activity of EBV DNA polymerase was determined in the presence of 100 mM (NH₄)₂SO₄ as described in the text. The difference between control and serum neutralization activity was calculated and the anti-EBV DNA polymerase activity was expressed as the units of DNA polymerase neutralized by 1 ml of undiluted serum.

Fig. 6. Relationship between serum titers of anti-EBV DNA polymerase and anti-EBV DNase. The serum titers of antibody to EBV DNase were assayed as described by Cheng et al. (14), where anti-DNase activity of less than 6 units/ml corresponds to a "normal" serum. The serum titers of antibody to EBV DNA polymerase were determined as described in the text, where serum values less than 180 units/ml corresponds to a "normal" serum.

62.0 ± 56 units/ml serum (mean ±SD). In the case of NPC, forty-eight sera were examined and the activity of EBV DNA polymerase neutralized was 380 ± 168 (mean ±SD). In comparison with that of healthy donors, the difference between these two groups is statistically very significant (p < 0.01). Taking neutralization activity of 0–180 units/ml serum (which was derived from the mean value of healthy donors ± 2 SD) as a normal range, values higher than 180 units/ml serum were considered as abnormal or positive. Two out of 52 sera from healthy donors were found in the abnormal titer range; one was 250, and another was 220 units/ml serum. Thus the false-positive rate was 3.8%. In contrast, 41 out of 48 sera from patients with NPC were higher than 180 units/ml serum; the highest titer was 660 units/ml serum. The positive rate of NPC serum was therefore 85.4% (Fig. 5).

Relation of Apparent Anti-EBV DNA Polymerase Activity with Titters of Other EBV-specific Antibodies in NPC Serum. As shown in Fig. 6, the activity of apparent anti-EBV DNA polymerase was plotted against the activity of anti-EBV DNase. Among the 40 sera samples from patients with NPC, 28 were positive both in anti-EBV DNA polymerase and anti-EBV DNase assays and one was negative for both of these parameters. In order to examine whether the titer of the serum IgA to VCA and IgA to EA(D) correlated with that of anti-EBV DNA polymerase, 12 NPC sera samples which had low IgA or low EA(D) titers were selected for study. The results are summarized in Table 3. In the 6 VCA-negative sera (VCA < 1:10, most of EA(D) were negative), 4 sera were positive for activity of anti-EBV DNA polymerase (>180 units/ml serum) (i.e., NPC-019, NPC-035, RNPC-001, and RNPC-002). In 6 other VCA-weak-positive sera, 4 strong positives with serum activity of anti-EBV DNA polymerase were observed (i.e., RNPC-004, RNPC-005, RNPC-006, and NPC-042). Therefore, the titer levels of antibodies to the VCA and EA(D) and anti-EBV DNA polymerase do not have complete correlation.

DISCUSSION

A salt-dependent DNA polymerase could be induced in Raji cells after treatment with TPA and n-butyrate. The induced EBV DNA polymerase appeared to be the same as the EBV DNA polymerase in P3HR-1 cells as supported by the following data. This purified enzyme was sensitive to phosphonoformate and had a salt optima similar to the EBV DNA polymerase from P3HR-1 cells. The salt-dependent enzyme induced was eluted in a similar fashion to that of EBV DNA polymerase from P3HR-1 cells through various steps of purification (4). This induced enzyme in Raji cells (nonproducer cell line) could be separated from its stimulating protein on a phosphocellulose column. This result is identical with that for the EBV DNA polymerase in P3HR-1 cells (producer cell line). During the course of the present study, the induction of EBV DNA polymerase in Raji cells treated with TPA and n-butyrate was reported by other investigators (23).

It was shown previously in this laboratory that high frequency and high levels of antibodies to EBV DNase are found in NPC sera. In this report, we also found that the induced EBV DNA polymerase, not only of the crude extract but also of the DEAE-cellulose, phosphocellulose, and DNA-cellulose purified preparations, could be neutralized by NPC serum (Fig. 3; Table 1). As to the immunospecificity of the serum antibody to EBV DNA polymerase, sera from patients with NPC could neutralize the EBV DNA polymerase in both induced Raji cells and induced P3HR-1 cells. Therefore, the EBV DNA polymerase in induced Raji cells had the same immunogenicity as that induced in P3HR-1 cells. The antibody to EBV DNA polymerase in NPC serum did not, however, neutralize the DNA polymerase of HSV-1 and HSV-2, suggesting that the antibody to EBV DNA polymerase in NPC serum is immunospecific.

Serum immunoglobulin in NPC serum could be separated into six peaks on a Protein A-Sepharose column. Peak 1, which

<table>
<thead>
<tr>
<th>Patient</th>
<th>VCA-IgA</th>
<th>EA(D)-IgA</th>
<th>EBV DNA polymerase activity neutralized (units/ml serum)</th>
<th>Clinical status</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPC-019</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>258</td>
<td>BT</td>
</tr>
<tr>
<td>NPC-022</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>100</td>
<td>BT</td>
</tr>
<tr>
<td>NPC-035</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>290</td>
<td>BT</td>
</tr>
<tr>
<td>NPC-037</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>140</td>
<td>BT</td>
</tr>
<tr>
<td>RNPC-001</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>305</td>
<td>REL</td>
</tr>
<tr>
<td>RNPC-002</td>
<td>&lt;10</td>
<td>10</td>
<td>420</td>
<td>REL</td>
</tr>
<tr>
<td>RNPC-003</td>
<td>10</td>
<td>10</td>
<td>123</td>
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<td>RNPC-004</td>
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<td>REL</td>
</tr>
<tr>
<td>RNPC-006</td>
<td>10</td>
<td>20</td>
<td>530</td>
<td>REL</td>
</tr>
<tr>
<td>NPC-011</td>
<td>10</td>
<td>10</td>
<td>82</td>
<td>BT</td>
</tr>
<tr>
<td>NPC-042</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>420</td>
<td>BT</td>
</tr>
</tbody>
</table>

*BT, before treatment; REL, relapse.*
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contained IgA, IgM, and IgG, did not neutralize the EBV DNA polymerase and was different from the serum antibodies to VCA or EA(D) which distributed both in IgA and IgG of NPC serum. Peaks IV, V, and VI, which were eluted by pH 4.5-3 buffer, had a strong ability to neutralize EBV DNA polymerase. According to the report by Duhamel et al. (22), these fractions might contain large amounts of IgG; and small amounts of IgM and IgA. No IgG was observed.

As to the titers of serum antibody to EBV DNA polymerase, those of 2 out of 52 healthy donors exceeded 180 units/ml serum. One of the positive donors had suffered from mononucleosis 6 months before collecting her first blood sample. Eight months after the first collection, the serum was taken and the titer was decreased to normal range. More extensive studies should be performed in examining whether this apparent anti-EBV DNA polymerase activity is altered in individuals with mononucleosis.

The mean value of titers for healthy donors was 62 units/ml serum but that of NPC sera was 380 units/ml serum, which was more than 6 times as high as that of healthy donors; the titers of 41 out of 48 NPC patients exceeded 180 units/ml serum. The positive rate was 85.4%. The unpublished data from this laboratory indicated that this titer may even be useful for early diagnosis of patients with NPC. Among patient sera examined, 70% of them had high serum titers of both anti-EBV DNA polymerase and anti-EBV DNase; 27.5% of sera samples examined had a high titer of either one (Fig. 6). If a positive is considered as a high titer of either anti-EBV DNA polymerase or anti-EBV DNase in all the sera samples, then the positive rate of all the NPC sera would be increased to 97.5% (39/40). Therefore, the combined use of titer values of anti-EBV DNA polymerase and anti-EBV DNase activity in serum might be worth exploring for use in early detection of NPC in patients at high risk for NPC. This will be further investigated. It should also be pointed out that the combined use of anti-EBV DNA polymerase and VCA/IgA serum levels may also be worth investigating. Previously, anti-VCA/IgA serum levels were shown by others to be useful for early diagnosis of NPC (24, 25). In this study, we observed a lack of correlation between titers of anti-EBV DNA polymerase and VCA/IgA (Table 3). Whether the serum titer of anti-EBV DNA polymerase activity could be useful for prognosis and for monitoring the response of NPC patients to treatment should also be investigated.

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