

Original Article

# Possible Role for External Environmental Stimuli in Nasopharyngeal NK/T-Cell Lymphomas in the Northeast of China with EBV Infection-Related Autophagic Cell Death : A Pathoepidemiological Analysis

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This study examined the relationship between external environmental factors and Epstein-Barr virus (EBV) infection in nasal natural killer (NK)/ T-cell lymphomagenesis. Archival paraffin sections from 134 cases of nasopharyngeal lymphomas in the northeast of China were investigated by *in situ* hybridization of EBV-encoded small RNA-1 (EBER-1) and by immunohistochemistry of the status of programmed cell death (PCD). The cases examined included 74 (55.2%) cases of NK/T-cell lymphomas (NKTCL) in T-cell and NK-cell neoplasms as well as 32 (23.9%) cases of B-cell neoplasms (B-MLs) and 9 (6.7%) cases of carcinomas. These cases indicated a significant dominant occurrence of NKTCL in the nasal cavity and of B-MLs in the pharynx. Many EBV-associated NKTCLs were seen in the nasopharynx, all three cases of EBV-associated B-MLs were in the nasal cavity and all three cases of EBV-associated carcinomas were only seen in the pharynx. The low number of NKTCL cases showing little or no EBV association, together with the existence of EBER-1-free lymphoma cells in EBV-associated NKTCLs, suggested EBV-related lymphoma cell expansion during lymphomagenesis. Peculiar necrosis, frequently observed in NKTCLs, was due to accelerated PCD. This PCD was autophagic cell death as judged by labeling of Beclin-1 and LC3, which possibly occurred due to EBV infection, when apoptosis was suppressed by survivin. Very minute squamous carcinomas, observed in 10 of 23 cases of NKTCLs with residual epithelia that were survivin-positive but not EBV-associated suggested that carcinogenesis occurred before lymphomagenesis. These data suggest that external environmental oncogenic factors initiate nasopharyngeal carcinomas and lymphomas whereas EBV infection promotes them. [*J Clin Exp Hematopathol* 49(2) : 97-108, 2009]

**Keywords:** nasal NK/T-cell lymphoma, pathoepidemiology, autophagic cell death, Epstein-Barr virus, external environmental factors

## INTRODUCTION

A nasal lymphoma was first reported in 1897 and named

rhinitis gangrenosa.<sup>1</sup> With further investigation of immunological phenotypes and genotypes of lymphoma cells, a dominant subtype of nasal lymphomas was shown to be of the extranodal natural killer (NK)/T-cell lymphoma (NKTCL) type.<sup>2,3</sup>

NKTCL, a clinicopathological subtype of NK-cell and T-cell neoplasms (T-MLs), is more common in Asian countries and is especially prominent in China,<sup>2,3</sup> including the northeast of China.<sup>4</sup> NKTCLs are mostly comprised of lymphomas of the CD56<sup>+</sup> NK cell lineage, rare lymphomas of the cytotoxic CD56<sup>-</sup> T-cell lineage<sup>2</sup> and polymorphous reticulosis.<sup>3</sup>

NKTCLs have been shown to be highly associated with infection by Epstein-Barr virus (EBV).<sup>5</sup> This associated EBV is predominantly subtype A due to the known geographic distribution of EBV subtypes in NKTCLs.<sup>3</sup> A low population of HLA-A\*0201-restricted cytotoxic T-lymphocytes against epitopes on the latent membrane protein (LMP)-1 of EBV

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may explain the high association of EBV with NKTCLs.<sup>6</sup> However, it is not clear if EBV is a causative agent for NKTCLs, although EBV infection may play a role in NKTCL development.<sup>3</sup>

Genetic alterations in tumor suppressor genes such as p53 and oncogenes such as c-kit, have been investigated in NKTCLs but the results were variable.<sup>3</sup> A close relationship between exposure to pesticides and the incidence of malignant lymphomas<sup>7-9</sup> including NKTCLs<sup>10</sup> was reported in an epidemiological analysis of life-style and environmental factors. External environmental factors could influence nasal lymphomagenesis by diffusion through the nasal lumen into the mucosa of the nasopharynx. The direct and indirect effects of external factors on the mucosa and the infiltrates induce EBV-associated NKTCLs and EBV-associated nasopharyngeal carcinoma.<sup>11</sup> Thus, neoplastic changes in the mucosa of the nasopharynx must be investigated in order to understand the oncogenic effects of external environmental factors when EBV latency 0 phase infection is detected by *in situ* hybridization (ISH) of EBV encoded small RNA-1 (EBER-1) on archival paraffin specimens.<sup>12</sup>

NKTCLs are characterized histologically by a so-called peculiar necrosis described as extensive coagulative necrosis.<sup>2</sup> Only half of the cases of NKTCLs that express Fas (Apo-1/CD95) and Fas ligand (FasL) have mutations in the *FAS* gene, suggesting some mechanisms for resistance to Fas-FasL-inducing apoptosis.<sup>13,14</sup> However, so far little is known about programmed cell death (PCD) in NKTCLs. The process of PCD has been implicated in apoptosis,<sup>15,16</sup> autophagy<sup>17</sup> and other death inducing cellular events.<sup>16</sup>

In order to analyze PCD and apoptosis in histological sections key molecules involved in these pathways must be analyzed. During apoptosis, cleaved caspase-3 is the key protein that induces the irreversible processes of apoptosis. Cleaved caspase-3 can now be labeled by immunohistochemistry (IHC).<sup>18</sup> IHC analysis of cleaved caspase-3 and of anti-apoptotic factors such as Flip (FLICE-inhibitory protein), Bcl-2, apoptosis-antagonizing transcription factors (AATF) and survivin can indicate the status of apoptosis in tissues in archival paraffin sections.<sup>18-22</sup> Survivin suppresses cleaved caspase-3 and effects on cell proliferation.<sup>19,20</sup> Survivin is suppressed by wild type p53 protein<sup>23</sup> and appears in neoplastic cells and fetal tissues<sup>19,20</sup> when genetic and epigenetic regulation of survivin expression is of minor importance in the initiation of oncogenesis.<sup>24</sup> In EBV-associated gastric carcinomas, up-regulation of survivin by LMP-2A was reported.<sup>25</sup>

Autophagy is a lysosomal degradation pathway that serves an adaptive role in the protection of cells against diverse pathologies.<sup>17</sup> Macroautophagy degrades long-lived proteins and organelles and the molecules involved in macroautophagy have been elucidated. Proliferation stimuli and a sufficient amount of amino acids suppress the macroautophagy-

induction step in which Atg13, Atg17 and Atg1 form a complex.<sup>17</sup> Autophagic vesicle nucleation, occurring through a complex of Atg6/Beclin-1, Atg14, Vps34 and Vps15 is suppressed by a combination of Bcl-2/Bcl-X<sub>L</sub> and Beclin-1<sup>17</sup> so that a cross-talk between apoptosis and macroautophagy occurs when Atg1 is over-expressed.<sup>26</sup> Vesicle elongation is regulated by the Atg8/LC3 system and the Atg12 system.<sup>17</sup> We have previously succeeded in labeling Beclin-1 in normal human gastric epithelia in archival specimens using a super-sensitive IHC.<sup>27</sup> In this study we labeled Beclin-1 and LC3 and analyzed the status of macroautophagy in NKTCLs.

This study aimed to determine the effects of external factors on lymphomagenesis of NKTCLs and the relationship between lymphomagenesis of NKTCLs and EBV infection. We further aimed to determine the status of PCD and macroautophagy in the NKTCL cells by analysis of cellular markers of these processes. These studies were carried out by analysis of archival nasopharyngeal biopsy specimens obtained in the northeast of China that has many cases of NKTCLs.

## MATERIALS AND METHODS

### Materials

Archival paraffin specimens of 134 cases of nasopharyngeal lymphoma and its regional lymphatic tissues dating from 2001 to 2006 were re-examined in Kagoshima University Graduate School of Medical and Dental Sciences. These specimens were originally clinically diagnosed by pathological examination as malignant lymphoma in the Department of Pathology, China Medical University located in Shenyang in the northeast of China. Other than age, sex and biopsy site, other clinicopathological and viroserological information was not available.

This study was performed with the approval of the Ethics Committee for epidemiological studies in Kagoshima University Graduate School of Medical and Dental Sciences and of the Ethics Committee for international co-operative studies in China Medical University.

### Typing of lymphomas

The lymphoma cases were mostly re-categorized according to the WHO classification<sup>28</sup> with the following exceptions. Cytotoxic T-cell lymphoma was treated as an entity of T-MLs. Polymorphous reticulosis, which has recently been defined,<sup>3</sup> was treated as an early NK/T-cell lymphoma (early NKTCL). Among B-cell neoplasms (B-MLs), CD5<sup>+</sup> diffuse large B-cell lymphoma (DLBL) was differentiated from CD5-negative DLBL. Lymphoplasmacytic lymphoma, nodal marginal zone (monocytoid) B-cell lymphoma and a high grade case of marginal zone B-cell lymphoma (MzBL)-mucosa-associated lymphoid tissue (MALT) were categorized in

MzBL-MALT.

### *In situ hybridization (ISH) of EBER-1*

EBV infection was determined by ISH of EBER-1 that was performed according to a previously reported method.<sup>12</sup> Briefly, after deparaffinization, sections were digested with proteinase K at 37°C for 30 min, dehydrated, and dried. Hybridization with digoxigenin-labeled probes was then performed at 37°C more than three hr. The hybridized probes were visualized by means of alkaline phosphatase-labeled anti-digoxigenin antibody and a colorimetric alkaline phosphatase-Nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt activity reaction (DIG-nucleic acid detection kit, 1175041, Roche, Mannheim, Germany). After nuclear counterstaining with methyl green, sections were dehydrated and mounted in plastic medium.

An EBV<sup>+</sup> gastric adenocarcinoma, simultaneously stained by the same method, was used as positive control.

### *Antibodies used*

As listed in Table 1, the primary antibodies used for detection of the lymphoma cell phenotype in the archival paraffin sections were anti-CD3 $\epsilon$  (NCL-CD3-PS1/Vision Biosystems, Newcastle Upon Tyne, UK), CD5 (NCL-CD5-4C7/Vision Biosystems), CD79a (M7050/Dako, Glostrup, Denmark), TIA1 (TIA1/Coulter Immunology, Fullerton, CA, USA) and CD56 (NCL-CD56-IB6/Vision Biosystems).

The primary antibodies used for detection of the status of PCD and macroautophagy in the lymphoma cells were anti-cleaved caspase-3 (5A1 Asp175, Cell Signaling Co, Danvers, MA, USA), Flip (ab4042, Abcam, Cambridge, MA, USA),<sup>21</sup> survivin (ab469, Abcam),<sup>22</sup> AATF (ab39631, Abcam), Bcl-2

**Table 1.** Antibodies used and antigen retrieval/detection methods

Antibody	Specificity or function	Clone Source	Dilution	Antigen retrieval	Detection method
CD3 $\epsilon$	T cells, NK/T cells	NCL-CD3-PS1/ Vision Biosystems	1 : 100	Heat*	Polymer
CD5	T cells	NCL-CD5-4C7/ Vision Biosystems	1 : 50	Heat*	Polymer
CD79a	B cells	M7050/Dako	1 : 200	Heat*	Polymer
TIA1	T cells, NK/T cells (Cytotoxic granules)	TIA1/Coulter Immunology	1 : 500	Heat*	Polymer
CD56	NK/T cells, plasma cells	NCL-CD56-IB6/ Vision Biosystems	1 : 50	Heat*	Polymer
cleaved caspase-3	Irreversible apoptosis	5A1 Asp175/Cell Signaling Co.	1 : 200	Heat**	Polymer
FILP	Inhibitor of FADD, Caspase-8 and -10	ab4042/Abcam	1 : 50	Heat**	Polymer
Survivin	Inhibitor of apoptosis, suppresses cleavage of caspase-3 and -9, and is localized at microtubules in mitotic spindles during G2/M period.	ab469/Abcam	1 : 500	Heat**	Polymer
AATF/Che-1	Apoptosis-antagonizing transcription factor, enhances proliferation by interacting with Dik/ZIP kinase and RNA polymerase II and by binding Rb protein.	ab39631/Abcam	1 : 1000	Heat**	Polymer
Bcl-2	A mitochondrial membrane protein that stabilizes permeability of mitochondrial membranes and inhibits apoptosis	M0887/Dako	1 : 100	Heat*	Polymer
Beclin-1	Autophagic vesicle nucleation	Sc-11427/Santa Cruz	1 : 50	Enzyme	Supersensitive
LC-3	Autophagic vesicle elongation	0231s0104/Nanotools, PD014/MBL,	1 : 1000 1 : 1000	Heat** Heat**	Polymer Polymer
CD204/ SRA-E5	Macrophage scavenger receptor	Supplied from Prof. Takeya	1 : 1	Heat**	Polymer

Antigen retrieval methods : Heat\* : Sections were heated in citrate buffer pH6 (Target retrieval solution, S1699, Dako) for 5 min at 120°C in an autoclave. Heat\*\* : Sections in the citrate buffer (Diva Dechoaker, Biocare Medical) for 5 min at 120°C in an autoclave. Enzymatic antigen retrieval : Sections were treated with 200  $\mu$ g/mL proteinase K Tris buffer saline solution for 10 min at room temperature.

Detection methods : Polymer ; ChemMate Envision system (K5027, Dako). Supersensitive detection method ; The polymer method (K5027, Dako) with blocking of non-specific reactions, the catalyzed reporter deposition (CARD) reaction and detection in a catalyzed signal amplification (CSA) system with blocking diffusive deposition in CARD reaction<sup>29</sup>

(M0887, Dako), Beclin-1 (Sc-11427, Santa Cruz, Santa Cruz, CA, USA)<sup>27</sup> and LC3 (0231s0104, Nanotools, Teningen, Germany; PD014 and PM036, MBL, Nagoya, Aichi, Japan). These antibodies were used for staining of 40 archival paraffin sections that had an area free from degeneration. With the Beclin-1 antibody, Beclin-1 in the autophagic vesicle nucleation complex and mutated Beclin-1 in nucleus could be visualized without a non-specific stain in the cells of routinely formalin-fixed and paraffin-embedded tissue sections<sup>27</sup> by means of enzymatic antigen retrieval and supersensitive method<sup>29</sup> mentioned below. As for LC3, there is the processed form (LC3-I) and the vesicle membrane-bound form (LC3-II). Western blot analysis of the lyophilized cell lysate from serum starved Neuro 2A cells (supplied with 0231s0104 from Nanotools) with three anti-LC3 antibodies (0231s0104 from Nanotools, PD014 from MBL and PM036 from MBL) indicated that PD014 and PM036 labeled LC3-I and -II bands but 0231s0104 did not. In the ordinary formalin-fixed and paraffin-embedded sections of human lymph node with necrotizing lymphadenitis, PD014 and PM036 showed enough immunostaining in the ordinary sensitive IHC but 0231s0104 did not even in the supersensitive IHC.<sup>29</sup> PM036 showed low immunostaining in the antigen retrieval at pH6 and high immunostaining in the antigen retrieval independent of the pH of the solution mentioned below when PD014 revealed high immunostaining in both the antigen retrieval conditions. Thus, this study employed PM036 in the three antibodies against LC3. Since the immunostaining of the membrane-bound form LC3-II reflects the autophagic vesicle elongation, the specific evaluation of LC3 must be done in the background immunostaining of LC3-I.

Further, CD204<sup>+</sup> macrophages<sup>30</sup> in the 40 archival paraffin sections of lymphoma tissues were examined with the antibody supplied from Prof. Takeya M of Kumamoto University.

### ***Immunohistochemistry***

To retrieve antigen for detection of CD3 $\epsilon$ , CD5, CD79a, TIA1, CD56 and Bcl-2, sections were heated in the citrate buffer pH6 (Target retrieval solution, S1699, Dako) for 5 min at 120°C by means of an autoclave. To retrieve antigen for detection of cleaved caspase-3, Flip, survivin, AATF, LC3 and CD204, a heating method independent of the pH of the solution was performed in the citrate buffer solution (Diva Dechoaker, Biocare Medical, Concord, CA, USA) for 5 min at 121°C in an autoclave. Beclin-1 was retrieved by treating sections with 200  $\mu$ g/mL proteinase K (No. 9033, Takara Bio Co., Otsu, Shiga, Japan) -0.05M Tris buffer saline pH7.2 solution for 10 min.

For detection and visualization of the retrieved antigens except Beclin-1, the retrieved antigens were reacted with the appropriate specific primary antibody solution for 1 hr and

were visualized by means of a polymer method (ChemMate Envision, K5027, Dako) and a colorimetric diaminobenzidine (DAB)-peroxide (H<sub>2</sub>O<sub>2</sub>) reaction (DAB+ liquid system, K3468, Dako). Nuclear counterstaining was of hematoxylin stain (ChemMate hematoxylin, S2020, Dako). These procedures were performed by an autostainer (Autostainer, Dako) with rinsing buffer warmed at 35°C.<sup>29</sup>

For detection of the retrieved Beclin-1, after the second endogenous peroxidase inactivation in 3% H<sub>2</sub>O<sub>2</sub> phosphate buffer saline pH 7.2 for 5 min, the enzymatic retrieved Beclin-1 was reacted with the primary antibody solution for 15 min and were labeled with a polymer reagent (ChemMate Envision, K5027, Dako) for 15 min followed by catalyzed reporter deposition amplification reaction (K1500, Dako) with pretreatment against the non-specific reaction before each processes.<sup>29</sup> These procedures were performed by an autostainer (Autostainer, Dako) with rinsing buffer warmed at 35°C.<sup>29</sup>

Evaluation of the immunostaining was carried out on microphotographs of the immunostained slides to ensure absolute reproducibility of the evaluation process. The following graded scoring system (Score 0 : no labeled cells ; Score 1 : a few labeled cells ; Score 2 : some labeled cells ; Score 3 : many labeled cells ; Score 4 : most cells were labeled). The immunostaining of CD204 was evaluated as Score 4 when many CD204<sup>+</sup> macrophages formed a meshwork of their cytoplasmic processes. Staining of LC3 was evaluated by grading the cytoplasmic microgranular and macrogranular LC3 immunostaining as follows : Score 0 : no stain ; Score 1 : weak stain ; Score 2 : some stain ; Score 3 : strong stain ; Score 4 : very strong stain. Nuclear staining of LC3 was evaluated in cellular degenerative and degenerated areas.

## **RESULTS**

### ***Cases examined***

Lymphomas were typed based on immunostaining of the sections with the antibodies CD3 $\epsilon$ , CD5, CD79a, TIA1 and CD56. There were 69 (51.5%) cases of NKTCL, as indicated in Table 2 and these cases showed cellular (Fig. 1a), degenerative and degenerated areas. The degenerated areas exhibited peculiar coagulative necrosis with naked nuclear-like cellular debris. The NKTCL cells were CD3 $\epsilon$ <sup>+/+</sup> TIA1<sup>+</sup> CD56<sup>+</sup> medium to large-sized cells (Fig. 1b, 1c and 1e) and showed immunostaining of the neoplastic marker survivin in the cytoplasm and the nucleus (Fig. 1i). The CD204<sup>+</sup> macrophages formed a meshwork of their cytoplasmic processes in cellular areas (Fig. 1d, Table 3), were aggregated in degenerative areas, and were not visible in degenerated areas. There were rare intermingling small lymphocytes.

There were 5 (3.7%) cases of early NKTCLs (Table 2), defined recently by Aozasa.<sup>3,31,32</sup> Early NKTCLs showed a

**Table 2.** Epstein-Barr virus (EBV) infection status detected by *in situ* hybridization (ISH) of EBV-encoded small RNA-1 (EBER-1)

Diseases	Total (n = 134)	Nasal cavity (n = 82)	Pharynx (n = 38)	Others (n = 14)
	No. of cases (No. of cases with EBER-1 signal score 0:1:2:3)			
T-cell and NK-cell neoplasms	85 (7 : 2 : 5 : 71)	64 (5 : 2 : 3 : 54)	13 (2 : 0 : 2 : 9)	8 (0 : 0 : 0 : 8)
PTL unspecified	2 (2 : 0 : 0 : 0)	1 (1 : 0 : 0 : 0)	1 (1 : 0 : 0 : 0)	0
Cytotoxic T-cell lymphoma	9 (0 : 1 : 2 : 6)	5 (0 : 1 : 1 : 3)	3 (0 : 0 : 1 : 2)	1 (0 : 0 : 0 : 1)
NK/ T-cell lymphoma	74 (5 : 1 : 3 : 65)	58 (4 : 1 : 2 : 51)	9 (1 : 0 : 1 : 7)	7 (0 : 0 : 0 : 7)
Early NK/ T-cell lymphoma	5 (1 : 1 : 3 : 0)	4 (1 : 1 : 2 : 0)	1 (0 : 0 : 1 : 0)	0
NK/ T-cell lymphoma	69 (4 : 0 : 0 : 65)	54 (3 : 0 : 0 : 51)	8 (1 : 0 : 0 : 7)	7 (0 : 0 : 0 : 7)
B-cell neoplasm	32 (22 : 6 : 1 : 3)	8 (5 : 0 : 0 : 3)	19 (14 : 5 : 0 : 0)	5 (3 : 1 : 1 : 0)
B-lymphoblastic	3 (2 : 1 : 0 : 0)	0	2 (1 : 1 : 0 : 0)	1 (1 : 0 : 0 : 0)
Mantle cell lymphoma	1 (1 : 0 : 0 : 0)	0	1 (1 : 0 : 0 : 0)	0
DLBL	24 (18 : 4 : 0 : 2)	7 (5 : 0 : 0 : 2)	15 (11 : 4 : 0 : 0)	2 (2 : 0 : 0 : 0)
CD5 <sup>+</sup> DLBL	7 (4 : 2 : 0 : 1)	2 (1 : 0 : 0 : 1)	5 (3 : 2 : 0 : 0)	0
DLBL	17 (14 : 2 : 0 : 1)	5 (4 : 0 : 0 : 1)	10 (8 : 2 : 0 : 0)	2 (2 : 0 : 0 : 0)
MzBL-MALT	4 (1 : 1 : 1 : 1)	1 (0 : 0 : 0 : 1)	1 (1 : 0 : 0 : 0)	2 (0 : 1 : 1 : 0)
Composite lymphoma				
MzBL-MALT+				
Cytotoxic T-cell lymphoma	1 (0 : 1 : 0 : 1)	1 (0 : 1 : 0 : 1)	0	0
Hodgkin lymphoma	1 (1 : 0 : 0 : 0)	0	1 (1 : 0 : 0 : 0)	0
Cancers	9 (6 : 0 : 0 : 3)	5 (5 : 0 : 0 : 0)	4 (1 : 0 : 0 : 3)	0
Inflammation	3 (2 : 1 : 0 : 0)	2 (2 : 0 : 0 : 0)	1 (0 : 1 : 0 : 0)	0
Not-diagnostic	3 (3 : 0 : 0 : 0)	2 (2 : 0 : 0 : 0)	0	1 (1 : 0 : 0 : 0)

The graded scoring system for evaluation of the ISH of EBER-1 was : Score 0 : No positive cells. Score 1 : A few positive cells. Score 2 : Some positive cells. Score 3 : Many positive cells

PTL unspecified : Peripheral T-cell lymphoma, unspecified, NK/T-cell lymphoma : Extranodal natural killer/ T-cell lymphoma, nasal type, B-lymphoblastic : Precursor B lymphoblastic lymphoma, DLBL : Diffuse large B-cell lymphoma, MzBL-MALT : Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT), Composite lymphoma : Composite lymphoma of two different types

different histology by areas examined. The infiltrates in the surface mucosa comprised many CD3 $\epsilon$ <sup>+</sup> and/or TIA1<sup>+</sup> small lymphocytes and a few CD56<sup>+</sup> cells, were rich in CD204<sup>+</sup> macrophages and were positive for survivin. In the deep mucosa, CD3 $\epsilon$ <sup>+</sup> TIA1<sup>+</sup> CD56<sup>+</sup> (Fig. 1p) enlarged atypical lymphoid cells showed sinusoidal infiltration and were strongly positive for survivin (Fig. 1r) and CD204<sup>+</sup> macrophages showed the beginning of the formation of a meshwork of their cytoplasmic processes. Thus, early NKTCL showed 2 kinds of lymphoma cells, small CD3 $\epsilon$ <sup>+</sup> TIA1<sup>+</sup> cells in the surface mucosa and enlarged CD3 $\epsilon$ <sup>+</sup> TIA1<sup>+</sup> CD56<sup>+</sup> lymphoid cells in the deep mucosa.

There were 9 (6.7%) cases of cytotoxic T-cell lymphoma (Table 2). Also, there was a case of a cytotoxic T-cell lymphoma that existed as a composite lymphoma with a MzBL-MALT.

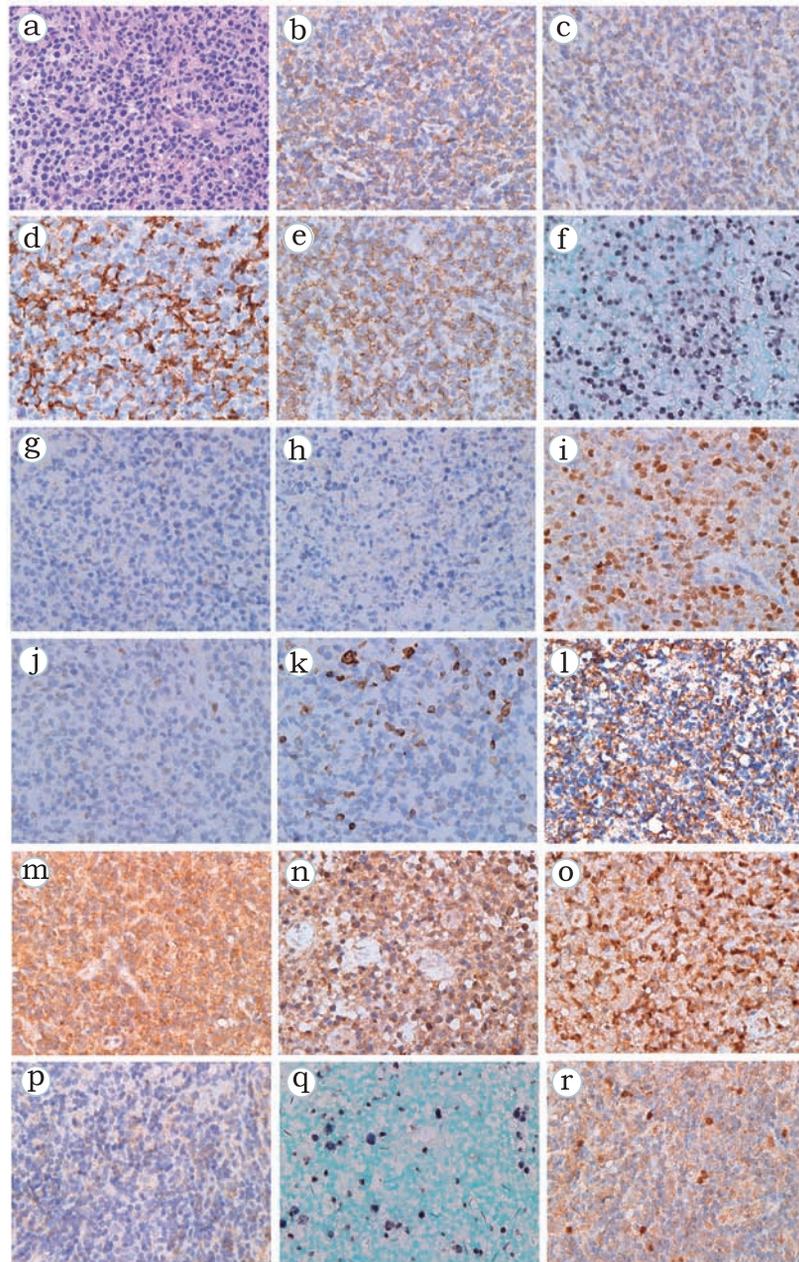
Among B-MLs, 7 (5.2%) cases of CD5<sup>+</sup> DLBL and one case of a high grade MzBL-MALT were differentiated from 17 (12.7%) other cases of DLBL (Table 2).

As well as lymphomas, there were 9 (6.7%) cases of carcinoma, 3 (2.2%) cases of inflammatory lesions, and 3 (2.2%) cases in which the quality of the specimens was inadequate for typing.

#### *Clinicopathological features of each type of lymphomas*

The male to female ratio (M : F) of patients with T-MLs was 1.77 (53 : 30, two cases unknown) and for patients with NKTCL was 2.05 (45 : 22, 2 cases unknown), indicating male dominance for these types of tumors. The male to female ratio of the total number of patients with B-MLs was 1.07 (16 : 15), of patients with CD5<sup>+</sup> DLBLs was 1.33 (4 : 3) and of patients with DLBL was 1.13 (9 : 8).

With regard to age, the mean and range (years) of the total number of patients with T-MLs were 45.6 (17-77), with NKTCLs were 43.8 (17-77), with B-MLs were 52.2 (13-84), with CD5<sup>+</sup> DLBLs were 56.9 (42-70) and with DLBLs were 54.6 (13-84). There was a significantly small number of



**Fig. 1.** Representative cases of natural killer/T-cell lymphomas (NKTCLs), from the nasal cavity of a 32-year old female (*1a-1o*) and an early NKTCL from the nasal cavity of a 65-year old female (*1p-1r*). (*1a*) Hematoxylin & Eosin stain, immunostaining of CD3 $\epsilon$  (*1b*), TIA1 (*1c*), CD204 (*1d*), CD56 (*1e*, *1p*), cleaved caspase-3 (*1g*, *1h*), survivin (*1i*, *1r*), Flip (*1j*), Bcl-2 (*1k*), Beclin-1 (*1l*), and LC3 (*1m-1o*), and EBER-1 ISH (*1f*, *1q*). The long axes of all the microphotographs ( $\times 40$  magnification) were 215  $\mu\text{m}$  long. The CD3 $\epsilon$ <sup>+</sup> TIA1<sup>+</sup> CD56<sup>+</sup> NKTCL cells (*1b*, *1c* and *1e*) show diffuse proliferation with a meshwork distribution of CD204<sup>+</sup> macrophages (*1d*), and EBER-1 signals in the cellular area (*1f*). There are no obvious cleaved caspase-3 positive cells in the cellular area (*1g*) or in the degenerative area (*1h*) although weak staining can be observed in some NKTCL cells (*1g*) and there is a visible stain in some degenerative macrophages (*1h*). Survivin is expressed in the cytoplasm of almost all of the NKTCL cells and in the nuclei of some NKTCL cells (*1i*). Only weak immunostaining of Flip is noted in the NKTCL cells (*1j*). Weak peri-nuclear immunostaining of Bcl-2 is seen in the NKTCL cells whereas intermingling B-cells show a strong stain (*1k*). Many NKTCL cells indicate microgranular immunostaining of Beclin-1 in the cytoplasm (*1l*) that is accompanied by strong microgranular staining of LC3 in the cytoplasm (*1m*). In the degenerative area, many NKTCL cells show strong macrogranular staining of LC3 in the cytoplasm (*1n*). In the degenerated area, LC3 appears to label naked or degenerated nuclei (*1o*). In the case of early NKTCL, there is sinusoidal infiltration of CD3 $\epsilon$ <sup>+</sup> TIA1<sup>+</sup> CD56<sup>+</sup> (*1p*) atypical lymphoid cells that exhibit both large and small EBER-1-positive nuclei (*1q*). These atypical lymphoid cells express survivin in the cytoplasm and, in some cells, also in the nucleus (*1r*).

**Table 3.** Status of programmed cell death

Diseases	Apoptosis					Autophagy (Macroautophagy)			
	CD204	Bcl-2	Flip	Survivin	Cleaved caspase-3	Beclin-1	LC3		
							Cyto*	Macro*	NS*
Mean score [Standard deviation (S.D.)]									
Cytotoxic T-cell lymphoma (n = 5)	2.6 (1.5)	0.6 (0.9)	1.2 (1.6)	3.6 (0.5)	0	1.2 (0.8)	3 (0)	2.6 (0.5)	2 (1.2)
EBV-NK/ T-cell lymphoma (n = 2)	2.5 (0.7)	4 (0)	1.5 (0.7)	3.5 (0.7)	0	3.0 (1.4)	3 (0)	1.5 (0.7)	0.5 (0.7)
Early NK/ T-cell lymphoma (n = 1)	3	2	1	3	0	2	3	1	0
EBV <sup>+</sup> NK/ T-cell lymphoma (n = 21)	3.9 (0.4)	0.8 (0.8)	1.2 (0.6)	4 (0)	0	2.1 (0.9)	2.9 (0.4)	3.2 (0.8)	2.8 (1.2)
CD5 <sup>+</sup> DLBL (n = 2)	1 (0)	3.5 (0.7)	1.5 (0.7)	3.5 (0.7)	0	2 (0)	3 (0)	3 (0)	1 (1.4)
DLBL (n = 6)	0.7 (0.5)	3.1 (0.9)	1.5 (0.5)	3.3 (0.5)	0	1.5 (0.5)	3 (0)	2.5 (0.5)	0.8 (0.8)
MzBL-MALT (n = 2 : EBV <sup>+</sup> )	0.5 (0.7)	1 (0)	1 (0)	3 (0)	0	1.5 (0.7)	3 (0)	3 (0)	2.5 (0.7)
Composite lymphoma (n = 1)									
MzBL-MALT	0	4	3	3	0	1	4	1	0
Cytotoxic T-cell lymphoma	3	2	1	3	0	2	4	1	0

The graded scoring system for evaluating CD204<sup>+</sup> macrophages : score 0 : no positive lymphoma cells, score 1 : a few positive lymphoma cells, score 2 : some positive lymphoma cells, score 3 : many positive lymphoma cells, score 4 : many lymphoma cells that form a meshwork of their cytoplasmic processes

The graded scoring systems for evaluation of the immunostaining of LC3:

Cyto\* : Cytoplasmic microgranular staining, Macro\* : Cytoplasmic macrogranular staining. The graded scoring system for Cyto\* and Macro\* ; score 0 : no stain, score 1 : weak stain, score 2 : some stain, score 3 : strong stain, score 4 : very strong stain

NS\* : nuclear stain. The graded scoring system for NS\* ; score 0 : no labeled nuclei, score 1 : a few labeled nuclei, score 2 : some labeled nuclei, score 3 : many labeled nuclei, score 4 : most nuclei were labeled

NK/T-cell lymphoma : Extranodal NK/ T-cell lymphoma, nasal type, EBV-NK/T-cell lymphoma : EBV-not-associated NK/ T-cell lymphoma, EBV<sup>+</sup> NK/T-cell lymphoma : EBV-associated NK/ T-cell lymphoma, DLBL : Diffuse large B-cell lymphoma, MzBL-MALT : Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT), Composite lymphoma : Composite lymphoma of two different types

patients with B-MLs that were under 40 years old ( $p = 0.028$ ).

A significant number of cases of T-MLs in the nasal cavity (64 cases), B-MLs in the pharynx (19 cases) (Table 2,  $p = 0.0000007$ ), as well as NKTCLs in the nasal cavity (58 cases) and DLBL in the pharynx (15 cases) (Table 2,  $p = 0.000002$ ) were recorded.

### ***Relationship between EBV infection and subtypes of nasal lymphoma***

Among the T-MLs examined, most cases of cytotoxic T-

cell lymphomas and NKTCLs were EBV-associated ones, based on EBER-1 ISH (Fig. 1f). However, in patients with early NKTCL both small and large EBER-1<sup>+</sup> nuclei were observed (Fig. 1q, Score 2 in Table 2) suggesting that there were two kinds and small numbers of EBV-infected cells in the early NKTCL cells. Among the cases of EBV-associated NKTCLs, some cases had a low score of EBER-1 ISH, suggesting also a small number of EBV-infected cells in the NKTCL cells. These data indicated an expansion of the EBV-infected cells during lymphomagenesis as was observed in the cytotoxic T-cell lymphomas.

Among B-MLs, all 3 EBV-associated cases were from the nasal cavity (Table 2,  $p = 0.019$ ). Notably, for MzBL-MALT, expansion of EBV-infected cells was detectable as a transformation to a higher grade.

The EBV-associated carcinomas (Score 3 in Table 2) were found only in the pharynx ( $p = 0.047$ , Table 1). One of the EBV-associated carcinomas was minute and invaded directly to the subepithelial tissue, suggesting a quite early phase of EBV-associated epipharyngeal carcinoma.

### ***Degenerative tendencies for the lymphoma tissues***

Degenerative tendencies of the lymphomas were evaluated histologically and based on the distribution of CD204<sup>+</sup> macrophages and the IHC for markers of apoptosis and autophagy. Circulatory disorders inducing necrosis of lymphoma tissue were noted in both T-MLs (5 cases) and B-MLs (4 cases) but a high number of cases with peculiar coagulative necrosis was only noted among cases of T-ML (61 cases in total, 5 cases of cytotoxic T-cell lymphomas and 56 cases of NKTCL,  $p = 0.0006$ ). One EBV-associated CD5<sup>+</sup> DLBL also showed sporadic foci of coagulative necrosis, suggesting a close relationship between EBV infection and coagulative necrosis.

NKTCLs showed a significantly higher distribution of CD204<sup>+</sup> macrophages than DLBLs (Welch's t-test:  $p = 0.042$ , Table 2), indicating again their symbiotic nature in NKTCLs (Fig. 1d) rather than the scavengers since CD204<sup>+</sup> macrophages were not visible in the degenerated areas.

Cleaved caspase-3 is a hallmark of apoptotic cells undergoing PCD. However, in lymphoma cells in which survivin was strongly expressed (Fig. 1i and 1r, Table 2) there were no obvious cleaved caspase-3 positive lymphoma cells (Fig. 1g and 1h). Some NKTCL cells showed very faint immunostaining of cleaved caspase-3 in the cytoplasm (Fig. 1g), indicating accelerated degradation of caspase-3.<sup>27</sup> Some degenerative and survivin-negative CD204<sup>+</sup> macrophages showed faint immunostaining of cleaved caspase-3 in the cytoplasm (Fig. 1h), suggesting that they were undergoing apoptosis. Regarding the presence of anti-apoptotic markers, EBV-associated NKTCLs had a significantly lower expression of Bcl-2 (Fig. 1k) than EBV-not-associated DLBL (Student's t-test,  $p = 0.000003$ , Table 3). Flip expression was low in almost all lymphomas (Fig. 1j) and none of the lymphomas examined expressed AATF. These data suggest that neoplastic expression of survivin suppressed apoptosis in the lymphomas while EBV infection suppressed the expression of Bcl-2.

We further assayed the markers of autophagy, Beclin-1 and LC3. The NKTCLs, even though they were associated or not with EBV [Student's  $p$  (one tail) = 0.036, Fig. 11, Student's t-test,  $p$  (one-tail) = 0.026], showed a higher expression of Beclin-1 in the cellular areas (Fig. 11) than the cyto-

toxic T-cell lymphomas (Table 3) when Beclin-1 expression was in the degenerative areas. LC3 was highly expressed in the cytoplasm of the lymphomas cells in almost all cases examined (Fig. 1m, Table 3). Higher macrogranular immunostaining of LC3 was found in lymphoma cells (Fig. 1n) of EBV-associated NKTCLs (Student's t,  $p = 0.006$ ) and cytoplasmic T-cell lymphomas (Student's t-test,  $p$  (one-tail) = 0.03) than in those of NKTCLs that were not associated with EBV. Furthermore, LC3 was labeled in more nuclei in the EBV-associated NKTCLs (Fig. 1o, Student's t-test,  $p = 0.016$ ) and in the cytotoxic T-cell lymphomas than in the EBV-not associated NKTCLs. These data suggest a close relationship between EBV infection and autophagic cell death.

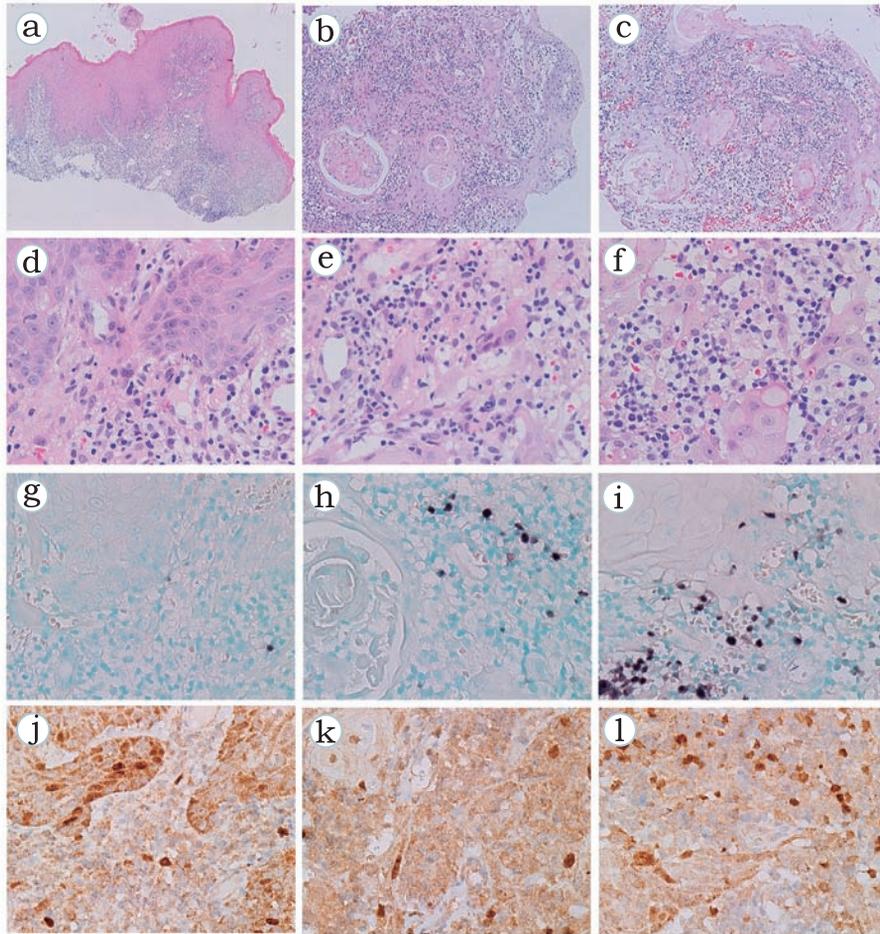
### ***Epithelial lesions that co-exist with nasal lymphoma***

To gain more insight into nasal lymphomas, we characterized any epithelial lesions that co-existed with the nasal lymphomas. Very minute epithelial lesions were found in only 26 (30.6%) of the T-MLs and in 7 (21.9%) of the B-MLs. These lesions were probably of pseudoepitheliomatous hyperplasia that has been documented in nasal type NKTCL.<sup>2</sup> Eleven of these cases showed a downward growth with elongation of the rete-ridge, dyskeratotic foci (Fig. 2a-2f) and immunostaining of survivin (Fig. 2j-2k). These characteristics suggested that the lesions were very minute squamous carcinomas.

Very minute squamous carcinoma showed a significantly higher association with T-MLs (11 out of 26 cases, Table 4,  $p = 0.04$ ), especially NKTCLs (10 out of 23 cases, Table 4,  $p = 0.04$ ), than with B-MLs (0 out of 7 cases, Table 4). Dysplasia and carcinoma also showed a higher association with T-MLs (18 out of 26 cases, Table 4) than with B-MLs (4 out of 7 cases, Table 4).

All 11 cases of the very minute squamous carcinomas were seen in the EBV-associated T-MLs. However, only two of these cases had a small number of EBER-1<sup>+</sup> carcinoma cells that closely localized with EBER-1<sup>+</sup> lymphoma cells. One case showed koilocytic change in some areas of the slide. The lymphoma cells that infiltrated into the carcinoma tissue were positive for either CD3 $\epsilon$  or CD56. Smaller NKTCL cells existed more in the subepithelial tissue than in the central areas, included only a small number of EBV-infected cells (Fig. 2g-2i), and showed a different histology from that of the early NKTCLs.

Furthermore, some survivin<sup>+</sup> cells could be classified as tissue stem cells and immature regenerative epithelia in the covering epithelia of the nasopharyngeal lymphomas. Beside fetal tissue, bone marrow tissue stem cells and neoplastic cells, survivin could appear in adult tissue stem cells and their young descendent of tissue stem cells, as CD117 did in the adult gastric non-neoplastic epithelia<sup>27</sup> and suggested also by the fact that the genetic and epigenetic regulation of survivin



**Fig. 2.** Occurrence of so-called pseudoepitheliomatous hyperplasia in a case of NKTL from the nasal cavity of a 51-year old female. Hematoxylin & Eosin staining (2a-2f) [(2a),  $\times 4$ ; (2b) & (2c),  $\times 10$ ; (2d)-(2f),  $\times 40$ ], *in situ* hybridization of Epstein-Barr virus-encoded small RNA-1 (EBER-1) (2g-2i,  $\times 40$ ), and immunostaining of survivin (2j-2l,  $\times 40$ ). (2a), (2d), (2g) and (2j) indicate a downward papillary lesion. (2b), (2e), (2h) and (2k) indicate a downward elongation of the rete ridge with dyskeratotic foci. (2c), (2f), (2i) and (2l) indicate a downward elongation of the rete ridge with dyskeratotic foci. The long axes of the microphotographs were 2,150  $\mu\text{m}$  long at  $\times 4$ , 860  $\mu\text{m}$  long at  $\times 10$ , and 215  $\mu\text{m}$  long at  $\times 40$ . The downward papillary epithelial lesion (2a) is associated with small infiltrates in the subepithelial tissue (2d). Only a few cells are positive for EBER-1 (2g). Both the epithelia and the infiltrates are positive for survivin (2j), indicating the neoplastic nature of the epithelia and the infiltrates, surely very minute squamous carcinoma and small cells-dominated area with rare EBER-1-positive NKTL cells in the EBV-associated NKTL. The two epithelial lesions of downward elongation of the rete ridge with dyskeratotic foci are minute (2b and 2c). The NKTL cells show infiltrative proliferation among the elongated rete ridges (2e and 2f) with only some EBER-1 positive NKTL cells (2h and 2i). Both the epithelia and the NKTL cells are positive for survivin (2k and 2l). The two epithelial lesions are also very minute squamous carcinomas.

expression is of minor importance in the initiation of oncogenesis.<sup>24</sup>

## DISCUSSION

The neoplastic nature of the nasal type of NKTL is generally determined by analysis of the immunological phenotype to distinguish between mono- or oligo-types, analysis of the genotype of lymphoma cells, and by ISH detecting EBV EBER-1.<sup>33</sup> Based on the IHC of survivin, two candidate lymphoma cells that were CD3 $\epsilon^+$  CD5 $^{+/-}$  TIA1 $^+$  cytotoxic T-

cells in the surface mucosa and CD3 $\epsilon^+$  TIA1 $^+$  CD56 $^+$  NK/T-cells in the deep mucosa could be classified as early NKTLs since both cytotoxic T-cell lymphomas and NKTLs are categorized as nasal type NKTLs in the WHO classification.<sup>2</sup> However, *de novo* occurrence of cytotoxic T-cell lymphoma was suggested in the composite lymphoma with MzBL-MALT. The IHC of TIA-1 and CD56 was stable enough to be used for the differentiation of cytotoxic T-cell lymphomas from NKTLs. On the other hand, expansion of EBER-1 $^+$  (EBV-infected) lymphoma cells after the neoplastic changes

**Table 4.** Occurrence of epithelial lesions in the lymphomas

Diseases	Total (n = 134)	Nasal cavity (n = 82)	Pharynx (n = 38)	The others (n = 14)
	No. of cases and type of epithelia Total No. (normal or hyperplastic : dysplastic : carcinoma)			
T-cell and NK-cell neoplasms	85 (8 : 7 : 11)	64 (4 : 5 : 10)	13 (3 : 2 : 1)	8 (1 : 0 : 0)
PTL unspecified	2 (0 : 1 : 0)	1 (0 : 0 : 0)	1 (0 : 1 : 0)	0
Cytotoxic T-cell lymphoma	9 (0 : 1 : 1)	5 (0 : 0 : 1)	3 (0 : 1 : 0)	1 (0 : 0 : 0)
NK/ T-cell lymphoma	74 (8 : 5 : 10)	58 (4 : 5 : 9)	9 (3 : 0 : 1)	7 (1 : 0 : 0)
Early NK/ T-cell lymphoma	5 (1 : 1 : 1)	4 (0 : 1 : 1)	1 (1 : 0 : 0)	0
NK/ T-cell lymphoma	69 (7 : 4 : 9)	54 (4 : 4 : 8)	8 (2 : 0 : 1)	7 (1 : 0 : 0)
B-cell neoplasms	32 (3 : 4 : 0)	8 (0 : 0 : 0)	19 (3 : 3 : 0)	5 (0 : 2 : 0)
B-lymphoblastic	3 (0 : 0 : 0)	0	2 (0 : 0 : 0)	1 (0 : 0 : 0)
Mantle cell lymphoma	1 (0 : 0 : 0)	0	1 (0 : 0 : 0)	0
DLBL	24 (3 : 3 : 0)	7 (0 : 0 : 0)	15 (3 : 3 : 0)	2 (0 : 1 : 0)
CD5 <sup>+</sup> DLBL	7 (1 : 2 : 0)	2 (0 : 0 : 0)	5 (1 : 2 : 0)	0
DLBL	17 (2 : 1 : 0)	5 (0 : 0 : 0)	10 (2 : 1 : 0)	2 (0 : 1 : 0)
MzBL-MALT	4 (0 : 1 : 0)	1 (0 : 0 : 0)	1 (0 : 0 : 0)	2 (0 : 1 : 0)
Composite lymphoma				
MzBL-MALT + Cytotoxic T-ML	1 (1 : 0 : 0)	1 (1 : 0 : 0)	0	0
Hodgkin lymphoma	1 (0 : 0 : 0)	0	1 (0 : 0 : 0)	0
Cancers	9 (0 : 0 : 9)	5 (0 : 0 : 5)	4 (0 : 0 : 4)	0
Inflammation	3 (2 : 0 : 0)	2 (1 : 0 : 0)	1 (1 : 0 : 0)	0
Not-diagnostic	3 (2 : 0 : 0)	2 (2 : 0 : 0)	0	1 (0 : 0 : 0)

PTL unspecified : Peripheral T-cell lymphoma, unspecified, NK/T-cell lymphoma : Extranodal natural killer/ T-cell lymphoma, nasal type, B-lymphoblastic : Precursor B lymphoblastic lymphoma, DLBL : Diffuse large B-cell lymphoma, MzBL-MALT : Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT), Composite lymphoma : Composite lymphoma of two different types

labeled by survivin was suggested in the EBV-associated T-MLs and B-MLs, indicating a possibility that the neoplastic nature in EBV-associated lymphomas could be determined by EBER-1 ISH,<sup>33</sup> and the areas without expansion of EBV-infected lymphoma cells showed the original histogenesis of the lymphomas.

EBV-associated NKTCLs are believed to be neoplastic variants that arose during chronic active EBV infection (CAEBV),<sup>34</sup> However, on the basis of the data in this study, we suggest that NKTCL in the nasopharynx is a localized form of NKTCL with EBV-associated lymphomagenesis since most patients with the nasal type of NKTCL were free from immunological disorders.<sup>3</sup> CAEBV has not been diagnosed in the northeast of China although some pediatric cases have been reported in other areas of China. Therefore, the early phase of lymphomagenesis in nasal NKTCLs should be analyzed from viewpoints other than EBV infection.

The NKTCLs and cytotoxic T-cell lymphomas were characterized by the existence of peculiar coagulative necrosis. The status of PCD was analyzed in these lymphomas, and revealed suppression of Bcl-2 and Flip in EBV-associated

lymphomas. A previous study had reported the overexpression of Flip in the NKTCL cell lines,<sup>35</sup> indicating a possibility that NKTCL cell lines may not truly represent the situation in NKTCLs. Granzyme B/TIA1 leakage-induced apoptosis has been suggested as a causative event for coagulative necrosis in NKTCL.<sup>36</sup> However, in this study, apoptosis that was detected by labeling cleaved caspase-3 was suppressed by the neoplastic expression of survivin in NKTCL. The expression of Beclin-1 and the cytoplasmic microgranular immunostaining of LC3 indicated enhanced macroautophagy in NKTCLs. The suppression of Bcl-2 that suppresses Beclin-1 in the autophagic vesicle nucleation<sup>37</sup> may explain the enhanced macroautophagy in EBV-associated lymphomas. The events other than EBV infection were involved in autophagic vesicle nucleation, which was shown in the immunostaining of LC3, in EBV-not associated NKTCLs and DLBL that expressed Bcl-2 highly. The cytoplasmic macrogranular immunostaining of LC3 in the degenerative areas of the NKTCLs suggested an inhibition of cellular energy-dependent events. Enhanced cytoplasmic macrogranular immunostaining of LC3 indicated cellular energy-independent continuation of the au-

tophagic process after vesicle elongation had occurred. Nuclear immunostaining of LC3 in the degenerated areas, especially in the EBV-associated lymphomas, is a hallmark of autophagic cell death. Therefore, the observed peculiar coagulative necrosis with naked nuclear-like cell debris was taken as an indicator of autophagic cell death.

Pseudoepitheliomatous hyperplasia,<sup>2</sup> observed in NKTCLs and cytotoxic T-cell lymphomas was very minute squamous carcinomas based on positive immunostaining of survivin and the absence of EBER-1 signals. The occurrence of epithelial lesions, including these very minute squamous carcinomas, suggested a strong oncogenic effect of the external stimuli.<sup>38</sup> The effect of external oncogenic stimuli on the nasopharyngeal mucosa was significantly stronger for T-MLs than for the other lymphomas. The rare cases of B-MLs in patients in the age range of 20 to 39 years old suggested that external oncogenic stimuli exerted a very strong effect on cytotoxic T-cells and NK/T-cells. Thus, the accumulated findings in this study are as follows: TIA1<sup>+</sup> or CD56<sup>+</sup> infiltrates into the very minute squamous carcinomas, EBER-1-free cellular areas and survivin<sup>+</sup> small cellular areas in the EBV-associated NKTCLs, and rare intermingling small lymphocytes in the NKTCLs, could all be explained if the cytotoxic T-cell lymphomas and the NKTCLs originated in the cytotoxic T-cells and NK/T-cells that were part of the host reaction to the epithelial lesions. The secondary mucosal changes could then be explained as having occurred due to strong oncogenic external stimuli. Although most epithelial neoplasms are rejected by the immune response, some residual very minute squamous carcinomas may survive as shown in this study.

We believe that elucidation of the oncogenic external stimuli that impact the nasopharyngeal mucosa will contribute to preventive medicine not only against nasopharyngeal lymphomas but also against nasopharyngeal carcinomas.

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