The Chinese-German Journal of Clinical Oncology Aug. 2005, Vol. 4, No. 4 P238–P243
DOI 10.1007/s10330-004-0351-8

Analysis of Survivin Expression in the Subtypes of Lymphoma

GU Xia[1], LIN Hanliang[1], SHAO Jianyong[2], ZHANG Men[1], and LIANG Huizhen[1]

[1] Department of Pathology, Basic Medical College, Sun Yat-sen University, Guangzhou 510080, China
[2] Department of Pathology, Cancer Center, Sun Yat-sen University, Guangzhou 510080, China

Received: 27 December 2004 / Revised: 11 January 2005 / Accepted: 3 March 2005

Abstract ***Objective:*** To detect the expression of survivin in subtypes of lymphoma and its value in classifying subtypes of lymphoma. ***Methods:*** Paraffin-embedded samples collected from 219 cases of lymphoma and 13 cases of lymph node reactive proliferation from affiliated hospitals of Sun Yat-sen University during 2001–2003 were examined for the expression of survivin by using immunohistochemical staining. Reverse transcription-polymerase chain reaction (RT-PCR) was used to detect the mRNA expression of survivin in K562, HL60, Raji, and Jurkat cell lines. Semi-quantitative assay was used to evaluate the quantity of survivin protein and mRNA expression in subtypes of lymphoma. ***Results:*** Protein expression of survivin was high and strong in diffuse large B-cell lymphoma (DLBL) (88.6%, 70/79), Burkitt lymphoma (BL) (100%, 2/2), and lymphoblastic lymphoma (LBL) (92.3%, 12/13), while their expression was always lower and weaker in follicular lymphoma (FL) (18.2%), extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) (40.9%) and marginal zone lymphoma (MZL) (33.3%). There was a significant difference between the higher expression group (DLBL, BL and LBL) and lower one (FL, MZL, and MALT) in the expression of survivin (Chi-square test, χ^2=24.77, P<0.01). Almost all of Reed-Sternberg cells (R-S cells) in Hodgkin lymphoma (HL) strongly expressed survivin. The protein expression of survivin was positively correlated with mRNA (r=0.6270, P<0.01). ***Conclusion:*** The expression level of survivin mRNA and protein shows significant difference in subtypes of lymphoma. The expression of survivin mRNA might act as a biomarker to classify the subtypes of lymphoma.

Key words: survivin; lymphoma; classification; reverse transcription-polymerase chain reaction

It is difficult to diagnose and classify lymphoma in clinical pathology. To find a specific biomarker is an important way to overcome this problem. Survivin is a recently found member of the inhibitor of apoptosis protein (IAP) family. The distribution of survivin is different from Bcl-2. Survivin is undetectable in most normal adult tissues, but abundantly expressed in most human cancers[1] such as breast carcinoma, colon carcinoma, glioma and lung carcinoma[2−4]. In this study, immunohistochemistry and reverse transcription-polymerase chain reaction were used to detect the expression of survivin in subtypes of lymphoma and its significance.

Materials and methods

Cell culture

The cell lines of K562, HL60 (myeloid leukemia), Raji, Jurkat (lymphoma) and MCF-7 (breast cancer) were provided by our department. The cells of 1×10^7 were used to extract RNA, and some cells were collected to perform immunohistochemistry by cytospin slides.

Correspondence to: GU Xia. Department of Pathology, Basic Medical College, Xinjiang Medical University, Urumqi 830054, China. Tel: +86-991-4361313, Email: guxia1373@sohu.com

Patients and samples

Paraffin-embedded samples from 219 cases of lymphoma and 13 cases of reactive proliferation lymph node were collected from affiliated hospitals of Sun Yat-sen University during 2001–2003. Among them, there were fresh tissues from 18 cases of lymphoma and 2 cases of reactive proliferation lymph nodes. There were 137 males and 82 females, with a mean age of 52 years old (range 2–86). All samples were detected for CD5, CD10, CD20, CD23, CD38, CD43, CD45, CD45RO, Bcl-2 by immunohistochemistry, and the samples in some patients were stained for CD3, CD79, CD30. According to WHO 2001 classification, every section was reviewed by three pathologists again[5]. There were 79 cases of diffuse large B-cell lymphoma (DLBL), 11 cases of follicular lymphoma (FL), 21 cases of extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma), 6 cases of marginal zone lymphoma (MZL), 13 cases of lymphoblastic lymphoma (LBL), 2 cases of Burkitt lymphoma (BL), 4 cases of mantle cell lymphoma (MCL), 4 cases of small cell lymphoma (SLL), 28 cases of peripheral T-cell lymphoma (PTL), 22 cases of anaplastic large cell

Table 1 Expression of survivin protein in subtypes of non-Hodgkin lymphoma

Histological types	n	Expression level of survivin				Positive rate (%)
		–	+	++	+++	
Small lymphocytic lymphoma	4	3	1	0	0	25.0
Follicular lymphoma	11	9	0	2	0	18.2
MALT lymphoma	21	12	8	1	0	40.9
Marginal zone lymphoma	6	4	1	1	0	33.3
Mantle cell lymphoma	4	3	1	0	0	25.0
Diffuse large B-cell lymphoma	79	9	24	22	24	88.6
Lymphoblastic lymphoma	13	1	1	5	6	92.3
Peripheral T-cell lymphoma	28	4	8	6	10	85.7
Anaplastic large cell lymphoma	22	1	2	5	14	95.5
Burkitt lymphoma	2	0	0	1	1	100
Other types	8	1	1	2	4	87.5

Table 2 Ratio of survivin and β-actin OPTDI in leukemia and lymphoma cell lines

Cell lines	Types of histology	Survivin OPTDI/ β-actin OPTDI
MCF-7	Breast carcinoma	2.5335
K562	Myeloid leukemia	0.2931
HL60	Myeloid leukemia	1.0582
Raji	Burkitt lymphoma	1.2059
Jurkat	Lymphoblastic lymphoma	4.7196

lymphoma (ALCL), 21 cases of Hodgkin lymphoma (HL), and 8 cases of other types.

Reagents

SP kit (Zhongshan, China); Survivin (Santa Cruz, USA); CD5, CD10, CD23, CD43, Bcl-2 (ZAME, USA); CD20, CD38, CD45, CD45RO (Matxin, China); Trizol, primer and dNTP (Shengong, China); Reverse transcription kit (MBI, Lithuania); Taq polymerase (TaKaRa, Japan).

Immunohistochemistry

Paraffin-embedded samples were cut into sections for the evaluation of survivin protein expression. The method was to choice two points of specific pattern in HE stained sections. Using a boring machine to bore some tissue in the paraffin-embedded block, then put it into a hole in a new paraffin block to make up a tissue array. The tissue array block was done 4 μm thick tissue section. Immunohistochemical studies were carried out using a standard strepavidin (SP) technique. Briefly, before being labelled with anti-survivin antibodies, de-paraffinized and rehydrated tissue sections were overlain with 10 mM citrate buffer (pH 6.0) and heated in a pressure cooker while maintaining the pressure for 5 min. When the slides and buffer were at room temperature, the slides were rinsed in phosphate-buffered saline (PBS) and non-specific binding sites were blocked with 5% bovine serum albumin in PBS. After washing in PBS, primary anti-survivin antibody was diluted to 1:100 and incubated overnight at 4 °C. The samples were next incubated with biotinylated secondary antibody for 30 min at room temperature followed by strepavidin/biotinylation. The staining was preformed with 3,3-diaminobenzidine (DAB). Finally samples were counterstained with haematoxylin for 1 min and nuclei blued in water. Slides were then dehydrated and mounted. Negative control slides were made in the absence of primary antibodies, PBS instead of primary antibody. Positive control slides were cells of breast cancer cell line (MCF-7).

Extraction of total mRNA and reverse transcription

Total mRNA was extracted from cell lines and fresh tissues by Trizol reagent and reverse transcription was performed following the instructions of manufacturer.

PCR amplification

PCR amplification was performed on a PCR thermal cycler. The cDNA 1 μg mixture was subjected to amplification in a final volume of 50 μL containing 2 U Taq polymerase, 10×PCR buffer 5 μL, dNTP 1 μL, 10 μmol/L each of 5' and 3' primers. The conditions of PCR amplification were as follows: initial denaturation for 5 min at 95 °C, followed by 34 cycles of denaturation at 95 °C for 1 min, annealing at 57 °C for 1 min, extension at 72 °C for 1 min, and a finial extension at 72 °C for 5 min. The primer pairs of survivin (forward primer, 5'-TTCTTGGAGGGCTGCGCCT-3', reverse primer, 5'-CC TGGTAGTGGTGCAGCCA-3', 401 base pairs) and β-actin (forward primer, 5'-CCAAGGCCAACCGCGAGAA GATGAC-3', reverse primer, 5'-AGGGTACATGGTGGT GCCGCCAGAC-3', 587 base pairs) were used for amplification. Positive controls were MCF-7 for cell lines and K562 for fresh tissues. Normal human peripheral blood served as negative control.

Immunohistochemical staining for survivin and scoring

The expression of survivin protein was located at cellular nuclei, and some cytoplasm staining also was seen. It was positive cell that brown-yellow granule was detected in nuclei. According to the percentage of positive tumor cells,

Table 3 Expression of survivin mRNA and protein in tissues of lymphoma and reactive proliferation lymph nodes

Cases	Types of histology	Age	Sex	Survivin mRNA	Survivin protein
1	Diffuse large B cell lymphoma	42	Female	+++	+
3	Diffuse large B cell lymphoma	30	Female	–	–
5	Diffuse large B cell lymphoma	72	Male	++	+++
6	Diffuse large B cell lymphoma	70	Male	+	+++
7	Diffuse large B cell lymphoma	57	Male	+++	+++
8	Diffuse large B cell lymphoma	81	Female	++	+++
13	Diffuse large B cell lymphoma	41	Male	+	–
17	Diffuse large B cell lymphoma	52	Male	+++	++
16	Diffuse large B cell lymphoma	24	Male	++	–
18	Diffuse large B cell lymphoma	55	Female	+	–
2	Lymphoblastic lymphoma	19	Male	++	+++
4	Lymphoblastic lymphoma	20	Female	+++	+++
11	Lymphoblastic lymphoma	17	Male	++	++
9	Marginal zone lymphoma	70	Male	+	–
19	Marginal zone lymphoma	57	Male	+	–
15	Peripheral T cell lymphoma	63	Female	+	–
20	Peripheral T cell lymphoma	67	Female	+	++
14	Hodgkin lymphoma	14	Female	+++	R-S cell+
10	Reactive proliferation lymph node	13	Male	++	Follicular cells+
12	Reactive proliferation lymph node	41	Male	++	Follicular cells+

the expression of survivin protein was determined and assigned to one of the following 5 categories: 0 (<10%), 1 (11%–25%), 2 (26%–50%), 3 (51%–75%), 4 (>76%). The intensity of survivin immunostaining was scored as: 1 (light yellow), 2 (yellow) and 3 (brown). The percentage of positive tumor cells and staining intensity were then multiplied to produce a score: 0 (negative, –), 1–4 (weak, +), 5–8 (moderate, ++) and 9–12 (intense, +++).

Evaluation of RT-PCR

By using the cDNA of positive control MCF-7 cell line as temples, PCR amplification was performed for survivin and β-actin. One tube of PCR reaction was stopped from 18 cycles, and then each two cycles cutout one tube, until 38 cycles. Then the plateau phage was defined by electrophoresis in 2% agarose. The cycle number was the cycle number of plateau phage minus 2 cycles. The cycles for survivin and β-actin were 34 and 26 respectively. Using automatic image analysis system (KONTRON IBAS 2.0, Germany), the areas of electrophoresis bands multiplied mean optical density of the object (OPTDM), that can get integrated optical density of the object (OPTDI). According to ratio of survivin and β-actin OPTDI, the score was as follows: ≤0.0001 negative (–), 0.0001–0.5 weak positive (+), 0.5001–1 positive (++), ≥1 strong positive (+++). Semi-quantity assay was used to evaluate the quantity of survivin mRNA expression.

Statistical analysis

Statistical analysis was performed using rank sum test, Chi-square test and rank correlation. A P value less than 0.05 indicated statistical significance.

Results

Immunohistochemstry

Expression of survivin in K562, HL60, Raji and Jurkat cell lines Most cells (≥76%) were strained in nuclei. Protein expression of survivin was high and strong. It was clear to show nuclear profile, only a few cells were negative (Fig. 1).

Expression of survivin protein in subtypes of non-Hodgkin lymphoma Majority of survivin positive cells were tumor cells. Mesenchymatous cells (including small lymphocytes of non-tumor cells) often were negative for the expression (Table 1).

Expression of survivin protein was detected in DLBL (Fig. 2, 88.6%, 70/79). Many cells were high and strong positive (+++) (centreoblast and immunoblast). The media age of the patients positive for the expression of survivin was 55 years old, old than 45 years of the negative patients with DLBL. However, there was no statistical significance by rank correlation (P>0.05). The expression of survivin was detectable in LBL (Fig. 3, 92.3%, 12/13), and the expression in 46.2% (6/13) patients was high. However, the expression was always lower and weaker in FL (18.2%), MALT lymphoma (40.9%) and MZL (33.3%). There was significant difference between the higher expression group (DLBL, BL and LBL) and lower one (FL, MZL, and MALT) in the expression of survivin (Chi-square test, χ^2=25.42, P<0.01). Among 4 cases of MCL, one case was positive and the remaining were negative for the expression. Of the 4 cases of SLL, one was positive. In PTL and ALCL, the expression of survivin protein was high (85.7%, 24/28 and 95.5%, 21/22 respectively).

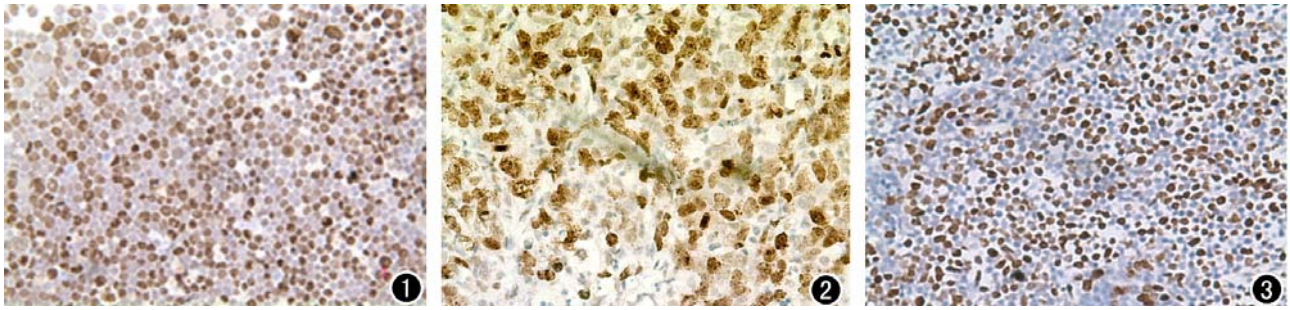

Fig. 1 Strong expression of survivin in K562 cell line (SP×200)
Fig. 2 Strong expression of survivin in nuclei of large cells (tumor cells) but negative in small cells (non-tumor cells) of DLBL (SP×200)
Fig. 3 Many positive cells for survivin in BL. Brown-stained granules located in nuclei of tumor cells (SP×400)

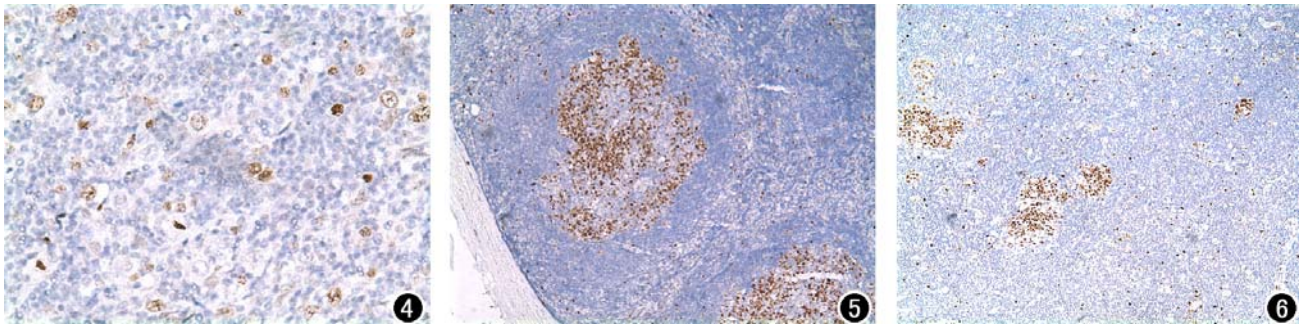

Fig. 4 Expression of survivin in nuclei of Hodgkin lymphoma's R-S cells and clearly showing the nuclear profile in R-S cells (SP×400)
Fig. 5 Positive expression of survivin protein in follicular centrocytic nuclei of lymph node with reactive proliferation (SP×40)
Fig. 6 Non-stained granules in all tumor cells of MCL. There was positive expression in nuclei of residual follicular centrocytes (SP×40)

Expression of survivin protein in HL Positive expression of survivin was detected in almost all of Reed-Sternberg cells (R-S cells) and the nuclear profile in R-S cells was shown, while non-tumor cells were negative (Fig. 4). The expression of survivin showed no difference in R-S cells between the mononucleated and multinucleated.

Expression of survivin in reactive proliferation lymph nodes High expression of survivin was seen in follicular germinal center cells of 13 lymph nodes with reactive proliferation and mantle and marginal zone cells were negative (Fig. 5). Meanwhile, the expression of survivin protein could clearly show residual follicular cells in some lymphoma (for example, MZL, MCL, Fig. 6).

RT-PCR detected the high expression of survivin in 4 cell lines

The electrophoresis band of survivin mRNA and β-actin were analyzed by image analysis system (Fig. 7). It was found that the ratio was high in Jurkat, while low in K562 (Table 2).

Survivin mRNA was amplified by RT-PCR in 18 cases of lymphoma and 2 cases of reactive proliferation lymph nodes. The RT-PCR products were separated by electrophoresis on 2% agarose and visualized with ethidium bromide. Using image analysis system, semi-quantity assay was used to evaluate the quantity of survivin mRNA expression (Table 3).

Survivin mRNA was detected in 9 DLBL cases, only one case was negative. From Table 3 it could be seen that case 3 was negative for survivin mRNA and case 6, 13 and 18 was weak positive. Strong positive rate was 60.0% (6/10). Survivin mRNA was detected in all 3 cases of LBL. In MZL, the expression was weak in 2 cases, and so was PTL. In 2 reactive proliferation lymph nodes, positive bands were seen. Strong positive expression was detected in 1 case of Hodgkin lymphoma (Fig. 8).

Relationship between survivin protein and mRNA

There was wide expression range (−−+++) for survivin either mRNA or protein in DLBL (Table 3), but the comparison in each case revealed that the expression intensity of both survivin mRNA and protein was coincident. The expression of survivin mRNA and protein was high (++−+++) in LBL. Survivin mRNA was weak expression in MZL, while its protein was negative by immunohistochemstry. The protein expression of survivin was positively correlated with mRNA (r=0.6270, P<0.01). Strong expression of survivin mRNA was de-

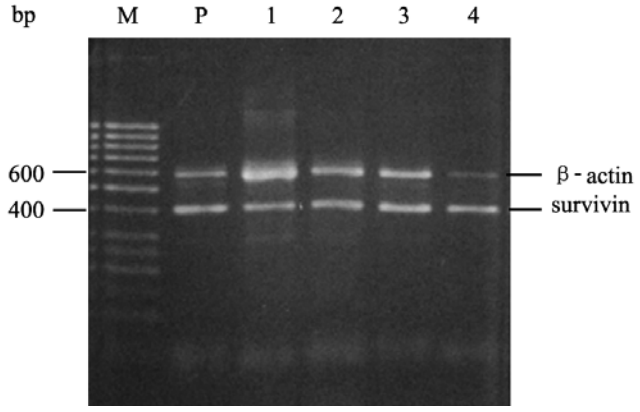

Fig. 7 Expression of survivin mRNA in 4 cell lines. M: Marker; P: Positive control (MCF-7); 1: K562; 2: HL60; 3: Raji; 4: Jurkat

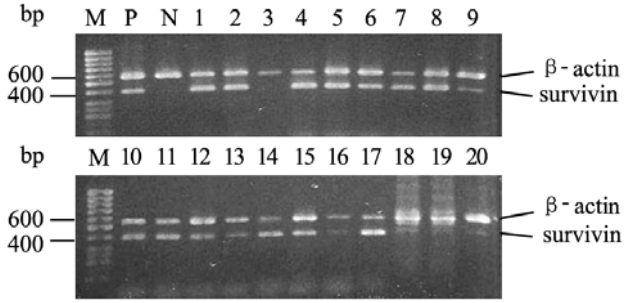

Fig. 8 Expression of survivin and β-actin mRNA in 18 cases of lymphoma and 2 lymph nodes with reactive proliferation. M: Marker; P: Positive control (K562); N: Negative control (peripheral blood lymphocytes in normal persons); 1, 3, 5, 6, 7, 8, 13, 16, 17, 18: DLBL; 2, 4, 11: LBL; 9, 19: MZL; 15, 20: PTL; 14: HL; 10, 12: Lymph nodes with reactive proliferation

tected by RT-PCR and R-S cells were stained by immunohistochemstry in one case of HL with mixed cellularity. In 2 reactive proliferation lymph nodes, positive survivin mRNA bands were seen and high protein expression detected in follicular germinal center cells.

Discussion

Survivin gene is located on chromosome 17q25, encoding a protein containing 142 amino acids, with a molecular weight of 16.5 KDa. Survivin was recently identified as a smallest molecular weight protein in IAP family, and it is characterized by a unique structure. Survivin was reported to bind directly to inhibit caspase-3 and caspase-7, to combine p21 indirectly to inhibit caspase, and to prevent apoptosis[6, 7]. Survivin is regulated by a cell cycle-dependent manner in phage G2/M and associated with spindle microtubules in mitosis[8]. This is different from Bcl-2 pathway of apoptosis.

It has been reported that survivin is a protein of cytoplasm. Positive expression of survivin was located at cytoplasm by immunohistochemistry and is associated with poor prognosis[9]. However, the expression of survivin was localized to the nuclear of tumor cells in our test. Rodriguez *et al*[10] reported survivin is a nuclear shuttling protein that is actively exported from the nucleus to cytoplasm. It is the important function of survivin that exported from nuclear to cytoplasm. The carboxy-terminal domain of survivin is necessary and sufficient for the nuclear-cytoplasmic localization of survivin protein. When this region is partially deleted, such as survivin-Ex3 with the three exon deletion, it leads to dysfunction of shuttling receptor, which is responsible for the strong accumulation of survivin-Ex3 in the nucleus. It is different significance that survivin protein is located at nuclear and cytoplasm. Although, some people reported that the expression of survivin in the nucleus was predictive of a favorable prognosis[11], majority was still reported that nuclear expression of survivin was associated with aggressive behavior and poor prognosis[10, 12, 13]. Our test supported the latter.

K562 and HL60 are cell lines of myeloid leukemia, Raji is cell line of Burkitt lymphoma, and Jurkat is cell line of lymphoblastic lymphoma. Their protein and mRNA survivin was high expression, especially in 2 lymphoma cell lines. It is suggested that high expression of survivin often exists in haematopoietic tumor, which was coincided with other reported[14].

Expression of survivin protein and mRNA were high and strong in DLBL. There was coincident with the expression of survivin mRNA and protein, and correlation between them. DLBL is a tumor of aggression, high proliferation and low apoptosis; meanwhile it was found that survivin protein expression level in older patients with DLBL was higher than that in younger patients. Although there was no statistical significance by rank sum test, the change tendency was obvious in them. Many people reported that high expression of survivin was associated with aggression of clinical procedure and poor prognosis[11, 12]. Therefore, it was thought that there was poor prognosis in older patients of DLBL. Although there were only 2 caes of BL, survivin was high expressed as the same as Raji cell line, suggesting that survivin gene played a role in apoptosis of BL. Majority of LBL occurs in adolescents. It usually presents at an advanced stage and is characterized by a highly aggressive behavior. Survivin mRNA and protein were high expressed. The expression level of survivin was lower and weaker or no expression in indolent lymphoma. Although MCL is historically considered an indolent variety of lymphoma, it has a relatively aggressive course and bad prognosis. Expression of survivin was lower in 4 MCL cases, which maybe related to slower course of the disease progression. Because of only 4 MCL cases, it is necessary to study further with more cases. Scholar[1] reported that the positive cells of survivin were often located to centroblast, immunoblast and lymphoblast, and matched to high expression of survivin in DLBL, BL and LBL. In 2 cases of FL positive for the expression of survivin, the histology was grade Ⅲ and most of positive cells were centroblast in FL, suggesting that

the apoptosis rate was lower and prognosis was worse in FL of grade Ⅲ than in grade I. Our results suggest that detecting the expression of survivin mRNA or protein and studying its intensity of expression was useful for classification of NHL.

The positive expression of survivin was detected in almost all of Reed-Sternberg cells (R-S cells), while non-tumor cells were negative. Our results suggest that survivin expression would be useful to diagnose HL.

Survivin is undetectable in most normal adult tissues, but abundantly expressed in embryo tissues and most human cancers. Because there were many transforming cells in germinal center of lymph node, including centroblast and immunoblast, it is suggested that survivin may play a role in lymphocytic transformation. Thus, combining survivin with Bcl-2 might aid to differentiate lymph node with reactive proliferation and follicular lymphoma, and to differentiate residual follicular cells in MZL and MCL.

References

1. Ambrosini G, Adida C, Altieri D. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med, 1997, 3: 917–921.
2. Nasu S, Yagihashi A, Izawa A, *et al*. Survivin mRNA expression in patients breast cancer. Anticancer Res, 2002, 22: 1839–1843.
3. Kawasaki H, Toyoda M, Shinohara H, *et al*. Expression of survivin correlates with apoptosis, proliferation, and angiogenesis during human colorectal tumorigenesis. Cancer, 2001, 91: 2026–2032.
4. Chakravarti A, Noll E, Black PM, *et al*. Quantitatively determined survivin expression level are of prognotic value in human gliomas. J Clin Oncol, 2002, 20: 1063–1068.
5. Jaffe ES, Harris NL, Stein H, *et al*. Pathology and genetics of tumors of haematopoietic and lymphoid tissues. WHO Classification of Tumors, 2001, 109.
6. Suzuki A, Ito T, Kawano H, *et al*. Survivin initiates procaspase 3/p21 complex formation as a result of interaction with Cdk4 to resist Fas-mediated cell death. Oncogene, 2000, 19: 1346–1353.
7. Wang XJ, Dai GY, Zhao XP, *et al*. Construction and characterization of an antisence RNA eukaryotic expression vecter for survivin. Chinese-German J Clin Oncol, 2003, 2: 246–249.
8. Li F, Ambrosini G, Chu EY, *et al*. Control of apoptosis and mitotic spindle checkpoint by survivin. Nature, 1998, 396: 580–584.
9. Lu CD, Altieri DC, Tanigawa N. Expression of novel anti-apoptosis gene, survivin, correlation with tumor cell apoptosis and p53 accumulation in gastric carcinomas. Cancer Res, 1998, 58: 1808–1812.
10. Rodriguez JA, Span SW, Ferreira CG, *et al*. CRM1-mediated nuclear export determines the cytoplasmic localization of the antiapoptotic protein survivin. Exp Cell Res, 2002, 275: 44–53.
11. Okada E, Murai Y, Matsui K, *et al*. Survivin expression in tumor cell nuclei is predictive of a favorable prognosis in gastric cancer patients. Cancer Lett, 2001, 163: 109–116.
12. Takai N, Miyazaki T, Nishida M, *et al*. Survivin expression correlates with clinical stage, histological grade, invasive behavior and survival rate in endometrial carcinoma. Cancer Lett, 2002, 184: 105–116.
13. Grabowski P, Kuhnel T, Muhr-Wilkenshoff F, *et al*. Prognostic value of nuclear survivin expression in oesophageal squamous cell carcinoma. Br J Cancer, 2003, 88: 115–119.
14. Moriai R, Asanuma K, Kobayashi D, *et al*. Quantitative analysis of the anti-apoptotic gene survivin expression in malignant haematopoietic cells. Anticancer Res, 2001, 21: 595–600.