Correlation of Seven Biological Factors (Hsp90α, p53, MDM2, Bcl-2, Bax, Cytochrome C, and Cleaved caspase3) with Clinical Outcomes of ALK+ Anaplastic Large-cell Lymphoma*

LI Hui Ling1,△, HUANG Xue Ping1,2,△, ZHOU Xin Hua2, JI Tian Hai2, WU Zi Qing2, WANG Zhi Qiang2, JIANG Hui Yong2, LIU Fan Rong2, and ZHAO Tong1,2,*

1.Department of Pathology, Nanfang Hospital affiliated to Southern Medical University, Guangzhou 510515 Guangdong, China; 2.Department of Pathology, School of Basic Medical Science, Southern Medical University, Guangzhou 510515, Guangdong, China

Abstract

Objective To explore correlation of seven apoptosis-related proteins (Hsp90α, p53, MDM2, Bcl-2, Bax, Cytochrome C, and Cleaved caspase3) with clinical outcomes of ALK+ anaplastic large-cell lymphoma (ALCL).

Methods Using immunohistochemistry and immunofluorescence double staining methods, the expressions of these seven apoptosis-associated proteins were studied to clarify their relationship with clinical outcomes of 36 ALK+ and 25 ALK- systemic ALCL patients enrolled between 1996 and 2006. The relationship of these apoptosis-regulating proteins with NPM-ALK status was also evaluated with the tyrosine inhibitor herbimycin A (HA) in vitro by immunocytochemistry, Western blotting and flow cytometric assays.

Results The presence of Hsp90α-, MDM2-, Bax-, Cytochrome C, and Cleaved caspase3-positive tumor cells was found significantly different in ALK+ and ALK- ALCLs, which was correlated with highly favorable clinical outcome. The Bcl-2- and p53-positive tumor cells were found in groups of patients with unfavorable prognosis. Inhibition of NPM-ALK by HA could reactivate the p53 protein and subsequent apoptosis-related proteins and therefore induced apoptosis in ALK+ ALCL cells.

Conclusion Our results suggest that these seven proteins might be involved in apoptosis regulation and associated with clinical outcome of ALK+ systemic ALCLs. We also reveal a dynamic chain relation that NPM-ALK regulates p53 expression and subsequent apoptosis cascade in ALK+ ALCLs.

Key words: ALK+ALCL; NPM-ALK; Prognostic factors; Apoptosis; Herbimycin A

INTRODUCTION

ALK+ ALCL is a distinct type of T-cell non-Hodgkin lymphoma characterized with unique morphologies and uniformly expressed CD30 antigen[1]. Most ALK+ ALCLs carry the t (2;5)(p23;q35) translocations, resulting in overexpression of nucleophosmin-anaplastic lymphoma kinase (NPM-ALK)[2-3]. NPM-ALK is a constitutively active tyrosine kinase, which generates activation of a
variety of antiapoptotic signals as well as proliferative ones involving the phospholipase C-λ (PLC-λ), JAK-STAT, PI3K-AKT-mTOR, Ras-ERK pathways, among other things. While many of the apoptosis-related proteins are now well understood, much less is known about relationship of these proteins.

NPM-ALK regulates a dynamic equilibrium in which p53 is excluded from the nucleus and degraded. The p53 regulates a master tumor apoptosis pathway that is inactivated in most cancers, and in some cases as a result of mutation of p53 itself or increased expression of the negative regulator MDM2. The functional p53-induced apoptosis occurs in the absence of Cytochrome C release into the cytosol and is caspase-dependent. The p53 gene mutations are uncommon in ALK+ ALCL, but they are frequently expressed in ALCL tumors at a variable level. However, exertion on function of NPM-ALK depends on heat shock protein 90α (Hsp90α). Hsp90α is a highly conserved, ubiquitous molecular chaperone that is required for the stability and conformational maturation of a diverse group of client proteins, including receptor tyrosine kinases such as breakpoint cluster region-Abelson (BCR-ABL), and NPM-ALK. To our current knowledge, Hsp90 inhibition has direct antitumor activity in tumor cells, such as Hodgkin’s lymphoma and Rhabdomyosarcoma.

The cell apoptotic program can be divided into two major pathways. (1) apoptotic signals emanating from cell surface death receptors, leading to activation of the Caspase8; (2) various apoptotic signals from the nucleus or the cytoplasm which are sensed by the BCL-2 homologue 3 domain—only members of the BCL-2 protein family resulting in Bax- or Bak-mediated cytoplasmic release of mitochondrial Cytochrome C. Both pathways induce apoptosis via activation of effector caspases, particularly Caspase3 that exerts final execution of apoptosis through degradation of vital proteins involved in cell structure, signaling, cell cycle control, and DNA repair.

This study analyzes seven biologic factors (Hsp90α, p53, MDM2, Bax, Bcl-2, Cytochrome C, and Cleaved caspase3) previously reported in other separate studies. These seven proteins are involved in apoptosis regulation and associated with clinical outcome of ALK+ systemic ALCLs. Furthermore, the effects of herbimycin A (HA) in vitro, a tyrosine kinase inhibitor are also assessed in an attempt to investigate relationship of these apoptosis-related proteins with NPM-ALK.

MATERIALS AND METHODS

Patient Selection and Clinical Manifestations

Formalin-fixed, paraffin-embedded tumor biopsies of 36 ALK+ and 25 ALK- systemic ALCLs were retrieved from the files of the Nanfang Hospital affiliated to Southern Medical University, Fuzhou General Hospital of Nanjing Military Region and Shenzhen Nanshan Hospital (diagnosed between 1996 and 2006). We selected 14 ALK+ and 16 ALK- systemic ALCLs with complete follow-up data for study. Of all patients, paraffin blocks of the primary diagnostic biopsies were investigated by HE staining and immunohistochemical staining. Cases were classified according to the World Health Organization classification, and diagnostic immunophenotyping included CD30, CD15, CD68, ALK, T-cell markers (CD3, CD43, and CD45RO), and B-cell markers (CD20, PAX5, and CD79a). No ALCLs of B-cell phenotype or Hodgkin-like ALCLs were included in this study; nor ALCLs arising in immunocompromised patients. For each patient, the following characteristics were noted from the medical records: age at diagnosis, sex, B symptom, onset position, bone marrow involvement, Ann Arbor stage at presentation, IPI classification, therapy, achievement of complete remission, the occurrence of relapses, length of survival. Of these 61 patients fifty-six received polychemotherapy consisting of CHOP regimen (cyclophosphamide, doxorubicin, vincristine, prednisone) with or without involved field radiation; Two patients only received one course of CHOP chemotherapy and the rest did not receive any chemotherapy. The patients’ records and clinical data were investigated retrospectively.

Immunohistochemical Detection of Prognosis-related Proteins

Sections with 3 μm thick from the paraffin-embedded biopsies were stained using a standard 3-step biotinstreptavidin-horseradish peroxidase detection system. The characteristics of the primary antibodies in immunohistochemistry are summarized in Table 1. The level of expression was assessed semiquantitatively using the immunoreactive scoring (IRS) system. The IRS value was determined by considering the staining intensity, which was visually scored in four degrees: absent (0), weak (1), moderate (2), and strong (3). The percentage of positive staining tumor cells was graded as absent (0), weak (1%-25%), moderate (21%-50%), and strong (51%-100%). Tissue samples were classified...
into either IRS=0 (negative) or IRS>0 (positive) staining. All antibodies required antigen retrieval by autoclave for 10 min in a citrate buffer (10 mmol/L, pH 6.0), after which sections were incubated for 2 h at room temperature (most antibodies) or incubated overnight (Bcl-2). Staining was visualized with 3,3-di-aminobenzidine tetrahydrochloride (DAB) substrate followed by hematoxylin nuclear counterstaining.

### Table 1. Immunohistchemistry: Characteristics of the Primary Antibodies

<table>
<thead>
<tr>
<th>Antibody Specificity*</th>
<th>Clone</th>
<th>Isotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALK</td>
<td>5A4</td>
<td>G1</td>
</tr>
<tr>
<td>Bcl2</td>
<td>C-2</td>
<td>G1</td>
</tr>
<tr>
<td>MDM2</td>
<td>SMP14</td>
<td>G1</td>
</tr>
<tr>
<td>p53</td>
<td>DO-1</td>
<td>G2a</td>
</tr>
<tr>
<td>Bax</td>
<td>B-9</td>
<td>G2b</td>
</tr>
<tr>
<td>Cleaved caspase3</td>
<td>7.1.44</td>
<td>G2a</td>
</tr>
<tr>
<td>Hsp90α</td>
<td>D7alpha</td>
<td>G</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>A-8</td>
<td>G2b</td>
</tr>
</tbody>
</table>

*Source: Hsp90α (Novus Biologicals, CO, USA), other antibodies (Santa Cruz, California, USA). Species: mouse.

### Immunofluorescence Double Staining

Immunofluorescence double staining was performed on formalin-fixed and paraffinembedded tissues. Antigen retrieval was performed by autoclave in 10 mmol/L sodium citrate buffer (pH 6.0) for 10 min. After cooling, slides were incubated in phosphate buffered saline (PBS) including 0.05% Tween20 for 5 min. Non-specific background was blocked by 5% human serum albumin (HSA) for 10 min. Anti-ALK was diluted in antibody diluent and incubated overnight at 4 °C. Anti-Hsp90α was incubated for 30 min at room temperature. Incubation with secondary antibodies was followed by three washing steps with PBS/Tween. Nuclei were counterstained by 5 min incubation with DAPI. The slides were examined with fluorescence microscope.

### Cell Culture

The human-derived NPM-ALK–expressing ALCL cell lines Karpas-299, SH-1 without NPM-ALK–expression and CML cell lines K562 were used for functional studies. Cell lines were cultured in RPMI 1 640 (Gibco-BRL, Beijing, China) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Gibco-BRL).

### Viability Measurement: Trypan Blue Exclusion Staining

For this assays, 3×10^5 cells from each cell line were cultured in duplicate 24-well tissue plates (COSTAR, NY, USA) and incubated with different concentration (0.25×10^-6 mol/L, 0.55×10^-6 mol/L, 1×10^-6 mol/L, 2×10^-6 mol/L, and 5×10^-6 mol/L, respectively) of herbimycin A (kindly provided by Pro. HUANG Wei Yi, Faculty of Life Sciences of Nanjing Agricultural College, Nanjing) for 72 h. Then trypan blue exclusion staining was used to detect cell viability. In a Neubauer chamber, cells were microscopically analyzed in duplicate for viability, which was then converted into inhibition rate. Each condition was performed in triplicate and all experiments were performed at least three times.

### Flow Cytometric Assays of cell Cycle and Apoptosis

For flow cytometric analysis, Karpas-299 cells were pretreated for 24 h with tyrosine kinase inhibitor (0.55 and 1×10^-6 mol/L Herbimycin A) or DMSO used as controls, if appropriate, followed by a 4 h fixation with 70% ethanol and 4h incubation with 100 μg/mL RNase (Sigma). After 3 h of staining with 50 μg/mL propidium iodide at 4 °C, flow cytometric analysis was done by flow cytometer (BECKMAN-COULTER, USA) using MULTYCYCLE software (PHEONIX, USA).

### Immunocytochemistry and Western Blotting Analysis

For the immunocytochemistry and Western blotting analysis of the proteins, Karpas-299 cells were pretreated every 12 h for 24 h with 0.55×10^-6 mol/L herbimycin A or DMSO. Immunocytochemistry was performed as described previously. The antibodies were used as follow: monoclonal mouse anti-phosphotyrosine (Clone P-Tyr-100, CST, Shanghai China), anti-Bcl-2, anti-MDM2, anti-p53, anti-Bax, anti-Cytochrome C; The polyclonal rabbit anti-Hsp90α.

Total proteins from the cell lysates were run on 8%-16% gradient ready-made sodium dodecyl sulfate polyacrylamide gel (Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride (PVDF). Blots were incubated with blocking solution (5% dry milk in TBST for 1 h). Primary antibodies anti-Alk and anti-cleaved caspase3 were used at dilution 1:200, and they were incubated overnight at 4 °C. Blots were subsequently incubated with secondary anti-igG-horseradish peroxidase-conjugated antibody.
Blots were developed using enhanced chemiluminescence kit and exposed to X-ray film.

**Statistical Analysis**

Correlation between the two groups was examined with the $\chi^2$ test and the Mann-Whitney U test. Overall survival (OS) was measured from the onset of treatment to the time of the last follow-up. Patient survival data were analyzed with the Kaplan-Meier method and were compared by means of the log-rank test. All $P$ values were based on two-tail statistical analysis, and $P$ values below 0.05 were considered significant. All analyses were performed using the SPSS statistical software package (version 13.0, SPSS Corporation, USA).

**RESULTS**

**Clinical Features**

Twenty males and ten females with an age range from 5 to 73 years (median age, 33.5 years) were enrolled in the study. Patients’ clinical manifestations and subgrouping according to ALK expression are summarized in Table 2. The ALK-positive group showed a dramatically younger age distribution in contrast to ALK-negative group (mean: 33.6±19.6 vs 57.0±17.2 years). The difference of sex between ALK-positive and ALK-negative cases was not statistically significant. There was a similar frequency of patients with either nodal-only disease or with multiple (more than one) extranodal sites of involvement. No differences in B symptoms or bone marrow involvement between ALK-positive and ALK-negative subgroups were observed. The incidence of the Ann Arbor stage had no difference. The international prognostic index (IPI) categories (low-low intermediate) of the ALK-positive group showed higher distribution than those of the ALK-negative group ($P$=0.011). For the ALK-positive group, the complete remission rate was 76.9% versus 33.3% for patients with ALK-negative tumors ($P$=0.024).

**Expression of Prognosis-related Proteins**

The results are summarized in Table 3 and categorized according to ALK+ and ALK- ALCLs. All of the ALK+ cases showed expression of Hsp90α, but the same expression was only in 4 of 16 (25%) ALK- cases. None of the ALK+ group showed any expression of p53, but most of them (93%) were positive for MDM2. The p53 was only positive in 4 of 16 cases (25%) of the ALK-group. Immunohistochemical profiles of p53-positive and MDM2-positive cases are shown in (Figure 1). Cleaved caspase 3 was positive in 10 of 14 (71%) ALK+ cases and in 5 of 16 (31%) ALK- cases, which was the same as the expression of Cytochrome C. In those ALCLs tested for active caspase 3, this effector caspase was detected as primarily nuclear staining of tumor cell (Figure 1AIII) and the percentage of active caspase 3-positive tumor cells ranged from less than 1% to 10%, with a mean of 3.5%. Bcl-2 was detected as cytoplasmic staining in tumor cells in 2 of 14 (14%) ALK+ ALCL cases and 13 of 16 (81%) ALK- group (Figure 1AV). When positive, the percentage of Bcl-2-positive tumor cells ranged from 2% to 80%, with a mean of 30%. Bax-expressing tumor cells were detected in 13 of 14 (93%) ALK+ ALCL cases and 7 of 16 (44%) ALK- group; staining was cytoplasmic (Figure 1AVI). In positive cases, percentages of Bax-positive tumor cells ranged from 5% to 70%.

Double labeling immunofluorescence showed colocalization of Hsp90α-immunoreactivity (Figure 1B, green fluorescence) and ALK-immunoreactivity (Figure 1B, red fluorescence) as displayed by the orange color in the overlay image.

---

**Table 2. Clinical Characteristics of ALCL Patients, According to ALK Expression**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>ALK+ (n=14)</th>
<th>ALK- (n=16)</th>
<th>$P^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (&lt;35/&gt;35)</td>
<td>12/2</td>
<td>2/14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sex (Male/Female)</td>
<td>9/5</td>
<td>11/5</td>
<td>0.799</td>
</tr>
<tr>
<td>Onset position(Lymph Node/Extranodal)</td>
<td>8/6</td>
<td>12/4</td>
<td>0.309</td>
</tr>
<tr>
<td>Bone marrow involvement(with/without)</td>
<td>2/12</td>
<td>5/11</td>
<td>0.281</td>
</tr>
<tr>
<td>B symptom (with/without)</td>
<td>9/5</td>
<td>12/4</td>
<td>0.530</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>11</td>
<td>8</td>
<td>0.111</td>
</tr>
<tr>
<td>III-IV</td>
<td>3</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

**International Prognostic Index**

| Low-low intermediate             | 11          | 5           | 0.011  |
| High intermediate-high           | 3           | 11          |        |
| Complete remission(with/without) | 10/3        | 5/10        | 0.024  |

**Note:** *As determined by the Mann-Whitney U test.*
Table 3. Expression of Prognosis-related Proteins

<table>
<thead>
<tr>
<th></th>
<th>ALK+ ALCLs (n=14)</th>
<th>ALK- ALCLs (n=16)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsp90α No. of positive cases (+/-)(%)</td>
<td>14/0 (100%)</td>
<td>4/12 (25%)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>p53 No. of positive cases (+/-)(%)</td>
<td>0/14 (0%)</td>
<td>4/12 (25%)</td>
<td>.048</td>
</tr>
<tr>
<td>MDM2 No. of positive cases (+/-)(%)</td>
<td>13/1 (93%)</td>
<td>9/7(56 %)</td>
<td>.026</td>
</tr>
<tr>
<td>Cleaved caspase3 No. of positive cases (+/-)(%)</td>
<td>10/4 (71%)</td>
<td>5/11 (31%)</td>
<td>.031</td>
</tr>
<tr>
<td>Cytochrome C No. of positive cases (+/-)(%)</td>
<td>10/4 (71%)</td>
<td>5/11 (31%)</td>
<td>.031</td>
</tr>
<tr>
<td>Bax No. of positive cases (+/-)(%)</td>
<td>13/1 (93%)</td>
<td>7/9 (44%)</td>
<td>.005</td>
</tr>
<tr>
<td>Bcl-2 No. of positive cases (+/-)(%)</td>
<td>2/12 (14%)</td>
<td>13/3 (81%)</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

Note. * Mann-Whitney U test for the comparisons between categorical variables.

Expression Levels of Hsp90α, p53, MDM2, Bcl-2, Bax, Cytochrome C, and Cleaved caspase3 are Related to Clinical Outcome

The overall survival curves of ALK+ ALCLs, shown in Figure 2A, demonstrated a significantly better survival for Hsp90α-positive ALCLs (P=0.046, log-rank test) (Figure 2A). Prognosis was found to be more favorable in 14 ALK+ cases with MDM2–positive tumor cells (P=0.024, log-rank test) (Figure 2B). When patients were divided into groups with and without Bax expression in tumor cells, the positive group was found with a significantly better survival (P=0.014) (Figure 2C). Similarly, the presence of Cytochrome C- and active caspase3-positive tumor cells identified groups of patients with a highly favorable prognosis (P=0.013 and 0.027, respectively) (Figure 2D and E).

When Bcl-2 and p53 were analyzed in a similar way, the prognosis was relatively poor in cases with...
ALK+ ALCL patient with 50% or more Bax–positive tumor cells (brown cytoplasmic staining). Original magnification for panels I-II was ×200 and III-VI was ×400. (B) Immunofluorescence double staining for Hsp90α and ALK. Hsp90α positive lymphoma cells (green) were also ALK positive (red), which was verified by a double exposure, where the cells appeared orange.

Bcl-2- and p53-positive tumor cells (P=0.014 and P=0.012, respectively) (Figure 2F and G).

**Herbimycin A Induces Cell-cycle Arrest and Apoptotic Cell Death in ALK+ ALCL Cells**

To investigate herbimycin A in the functional status of the apoptosis pathway, Karpas-299, SH-1, and K562 cell lines were treated with concentration gradient of herbimycin A for 72 h. Following this treatment, cells were stained with trypan blue exclusion staining. Obviously, there was a dose-dependent inhibition with herbimycin A in tyrosine-positive ALCL cells (Karpas-299) and K562 cell lines while ALK- ALCL cells (SH-1) had a minimal apoptotic response (Figure 3A).
Figure 2. Association of Hsp90α, MDM2, Bax, Cytochrome C, Cleaved caspase3, Bcl-2, and p53 with overall survival in patients with ALK+ ALCL groups. The patients with Hsp90α– (A), MDM2– (B), Bax– (C), Cytochrome C– (D) and Cleaved caspase3– positive (E) tumor cells showed better prognosis. The Bcl-2 and p53 positive groups showed significantly lower survival (F, G).
Figure 3. Herbimycin A induces cell-cycle arrest and apoptotic cell death in ALK+ ALCL cells. (A) Treatment with herbimycin A for 72 h resulted in a dose-dependent decrease in cell viability of Karpas299 and K562 cells measured by trypan blue exclusion staining, but not in SH-1 cells. (B) Histograms of the percentage of Karpas299 cells in each stage of the cell cycle after treatment with different doses of herbimycin A for 24 h were indicated above. The values of the mean of the percentage of cells from each group were also shown. (C) Detection of apoptosis by PI staining. Karpas299 cells were incubated with the herbimycin A at 0.55 and 1×10⁻⁶ mol/L drug concentrations for 24 h and apoptosis was determined by calculation of the subG₀/G₁ population following PI staining.

We assessed the response to herbimycin A by analyzing the DNA content of the Karpas-299 cells after different concentration of inhibitor (0.55 and 1×10⁻⁶ mol/L) was given. The percentage of cells in G₀/G₁ phase increased to 56.5%, whereas the percentage of cells in S phase decreased to 39.1% after 24 h of incubation with 0.55×10⁻⁶ mol/L herbimycin A compared with the controls (Figure 3B). The percentage of cells in G₀/G₁ phase rose to 67.3%, however, the percentage of cells in S phase declined to 29.1% after 24 h of incubation with 1×10⁻⁶ mol/L herbimycin A. These data suggested that although a single dose of herbimycin A was able to induce G₀/G₁ arrest within the initial 24 h of incubation, this was not sufficient to maintain the effect over the dose.

Typical results with herbimycin A analyzed for the apoptosis by PI staining was shown on the Karpas-299 cells in Figure 3C. Karpas-299 cells revealed markedly apoptotic response to herbimycin A, which was seen by PI staining as a sub-G₀/G₁ peak due to DNA fragmentation in cells undergoing apoptosis. Apoptosis rate of HA-treated Karpas-299 cells was 35.2% at 0.55×10⁻⁶ mol/L concentration and 56.2% at 1×10⁻⁶ mol/L concentration, compared with 5.2% in controls.

**Inhibition of Phosphotyrosine Kinase activity and ALK with Herbimycin A in ALK+ ALCL Cells**

It has been shown that the NPM-ALK-associated kinase activity involves autophosphorylation of tyrosine residues in the fusion protein[^19]. Some investigators have identified the relevance of the phosphorylation level of NPM-ALK in regulating its functional activity[^20-21]. To demonstrate functional inhibition of NPM-ALK in herbimycin A-treated Karpas-299 cell lines, protein extracts following incubation for 24 h were immunoprecipitated by anti-ALK antibody and subjected to Western blot analysis. NPM-ALK protein was absent in Karpas-299 cells after 24 h of incubation with herbimycin A compared with the controls (Figure 4A). Subsequent analysis of Western blotting demonstrated a notable quantitative decrease in the anti-ALK levels following inhibition of herbimycin A (Figure 4B). We also tested the inhibitory activity of herbimycin A on NPM-ALK associated autophosphorylation of tyrosine activity. As shown in Figure 4A, immunocytochemistry of karpas-299 cells exhibited a significant inhibition in phosphotyrosine after incubation with herbimycin A, indicating a decrease in both ALK levels and phosphotyrosine.

**Herbimycin A Activates Cleaved caspase3 in Karpas-299 Cells**

To examine the possible mechanisms underlying herbimycin A-mediated cell apoptosis, immunocytochemistry and Western blotting analysis was performed. With immunocytochemistry, its expression was strong and diffuse after a 24 h exposure to herbimycin A, but scattered in the control cells. The same result was validated by Western blotting in Figure 4B. Expression of Cleaved caspase3 as a result of exposure to herbimycin A was up-regulated in Karpas-299 cells.

**Effects of Herbimycin A on Hsp90α, MDM2, Wt-p53, and Apoptosis Regulatory Proteins**

We performed immunocytochemistry analysis to determine relative expression of Hsp90α, MDM2,
Wt-p53, Bax, Bcl-2, Cytochrome C. After 24 h drug treatment, the expression of both Hsp90α and MDM2 was markedly reduced in herbimycin A-treated Karpas-299 cells (Figure 4C). However, expression of both Bax and Cytochrome C was significantly increased in herbimycin A-treated cells. The wt-p53 protein was absent in the control cells but present in herbimycin A-treated cells after treatment of protease inhibitor for wt-p53 protein easily degrading. No significant changes in the expression of Bcl-2 were found in herbimycin A-treated Karpas-299 cells (Figure 4C).

**DISCUSSIONS**

In this study, clear clinicopathological differences were identified in 30 systemic ALCL patients with complete follow-up data between ALK-positive and ALK-negative types. ALK-positive ALCLs are characterized by a younger age distribution, earlier stage, lower IPI categories, and better prognosis. Moreover, expression of apoptosis-related proteins Hsp90α, p53, MDM2, Bcl-2, Bax, Cytochrome C, and Cleaved caspase3 in systemic ALK+ ALCLs are shown to be strongly related to ALK status, which is consistent with most of the studies in the literature[22]. We have also found that Hsp90α-, p53-, MDM2-, Bcl-2-, Bax-, Cytochrome C-, and Cleaved caspase3-positive tumor cells predict clinical outcome in systemic ALK+ ALCL cases. This is the first report evaluating seven biomarkers concurrently as prognostic factors in the same ALK+ ALCLs population.

The p53 gene, acting as a tumor suppressor gene, can be inactivated by a number of mechanisms including p53 gene mutation, the most frequent genetic alteration in human cancer[8], and increased expression of MDM2[23]. MDM2, a critical negative regulator of p53 that can regulate the p53 tumor suppressor by promoting its proteasome-mediated degradation resulting in cell cycle deregulation in ALK+ ALCLs. In a recent study, inhibition of the p53-MDM2 interaction by nutlin-3a can reactivate the p53 pathway resulting in cell-cycle arrest and apoptosis in ALK+ ALCL cells with wt or partially functional but mt p53[24]. To our knowledge, only the study by Rassidakis et al.[10] has assessed clinical outcome of MDM2 in systemic ALK+ ALCLs. This study has shown marked expression (93%) of MDM2 protein in ALK+ ALCL cases (Table 3), which is in relation to a highly favorable prognosis in ALK+ ALCL patients. However, p53 was not expressed in the ALK+ ALCLs. It is likely that, at least in a subset of these ALK+ ALCL tumors, increased levels of MDM2 can lead to increased degradation of p53.
Figure 4. Effects of herbimycin A on NPM-ALK-associated autophosphorylation activity and apoptosis-related proteins. (A) At 24 h after treatment of Kapars299 cells with 0.55×10^{-6} mol/L herbimycin A, expression of phosphotyrosine was decreased, even NPM-ALK was absent with immunocytochemistry. Reversely, expression of Cleaved caspase3 was strong and diffuse after a 24 h exposure to herbimycin A. (original magnification ×200). (B) Western blotting analysis at 24 h after treatment with herbimycin A showed decreased expression of NPM-ALK (p80) and increased expression of Cleaved caspase3 in Kapars299 cells. (C) Decreased levels of Hsp90α and MDM2 protein, in Kapars299 cells at 24 h after treatment with herbimycin A, were visualized using immunocytochemistry. However, expression of wt-p53, Bax and Cytochrome C was markedly increased in herbimycin A-treated cells. The expression of Bcl-2 remained unchanged (original magnification ×400).

In a recent study, Cui et al.\cite{25} has put forth an idea that NPM-ALK can enable degradation of p53 via both MDM2 and JNK activities. Accordingly, in this study the reactivation of potentially functional wt p53, by the tyrosine inhibitor herbimycin A, results in G0/G1 cell-cycle arrest and an increase apoptosis in ALK+ ALCL cells (Figure 3), which may reflect the characteristics of the intrinsic apoptotic pathway. The effect of herbimycin A down-regulates PI3-kinase by inhibiting NPM-ALK, subsequently leading to inhibition of phosphorylating and stabilizing MDM2, and results in the stabilization of p53. This activity is more pronounced in herbimycin A-treated cells, which display higher expression of wt p53 protein than in the control cells (Figure 4C).

Indeed, Hsp90 is vital to the survival of ALK+ ALCL cells, which enhances the stability of the NPM-ALK protein.\cite{26} Clinical outcome of Hsp90α is assessed in systematic ALK+ ALCL in our study for the first time. The present results have indicated that
Hsp90α overexpression has significant association with favorable outcome. We also showed the relation of Hsp90α and ALK status that Hsp90α was expressed in all of the ALK+ cases, but the same expression was only in 4 of 16 (25%) ALK- cases. Colocalization of Hsp90α and ALK in ALK+ ALCL tissues was also shown in our study (Figure 1B). When NPM-ALK expression was in vitro reduced in Karpas-299 cells by tyrosine inhibitor, expression of Hsp90α decreased dramatically (Figure 4). These data are in agreement with previous studies that NPM-ALK expression is strictly dependent on its interaction with Hsp90α.

It is known that Bcl-2 gene family functions as a regulator of the apoptotic pathway. Bcl-2 and Bax are likely the most important apoptosis regulatory proteins of this family. Bcl-2, which is a major negative regulator of apoptosis, was detected in only 2 (14%) of 14 ALK+ ALCL cases but in 13 (81%) of 16 ALK- ALCLs. These findings confirm previous studies by George et al. that NPM-ALK down-regulates Bcl-2 gene expression in vivo. The different expression of Bcl-2 in ALK+ ALCL cases may also be responsible for the differences in survival of patients. Notably, Bax was detected more frequently and at a significantly higher level (93%) in ALK+ systemic ALCLs, compared with 44% in ALK- ALCLs (Table 3). The imbalance between Bax and Bcl-2 may in part explain the susceptibility of ALK+ ALCL patients to chemotherapy. Drakos et al. have shown that proapoptotic protein Bax, known as transcriptional targets of p53, correlates with p53 expression and are found to increase in nutlin-3a-treated ALK+ ALCL cells. Here, we showed that exposure of NPM-ALK–expressing ALCL cells to the herbimycin A resulted in an increase in expression of Bax, with parallel activation of wt p53. These data are supporting the published study that wild-type p53 may induce expression of Bax. However, it is worth asking why expression of Bcl-2 was found unchanged in herbimycin A-treated tumor cells.

We also assessed the expression of the apoptosome components Cytochrome C and Cleaved caspase3 in ALCL tumors and found that Cleaved caspase3 was expressed in 71% of the ALK+ ALCL cases and 31% of the ALK- ALCL cases, which was the same as the expression of Cytochrome C. It is noteworthy that overexpression of Cytochrome C and Cleaved caspase3 may serve as a favorable prognostic factor in survival analysis. Furthermore, the different expression of Cleaved caspase3 in ALK+ and ALK- ALCLs may be due to differential expression of Bcl-2 family proteins in these tumors. Bcl-2 has been shown to inhibit the stress-induced apoptosis pathway by interfering with mitochondrial Cytochrome C release or by directly inhibiting the function of caspase3, which can rapidly activate the caspase3. It is also reported by Rosita L. ten Berge et al. that high caspase3 levels are correlated with ALK expression in systemic ALK+ ALCL tumors. Our study showed that expression of Cleaved caspase3, accompanied by Cytochrome C was notably up-regulated in herbimycin A-treated ALK+ ALCL cell lines.

In conclusion, the expressions of Hsp90α, p53, MDM2, Bcl-2, Bax, Cytochrome C, and Cleaved caspase3 are involved in apoptosis regulation and are associated with clinical outcome in systemic ALK+ ALCLs. This study is limited to the analysis of seven proteins, which were though previously reported in other separate studies, were devoid of their correlation with clinical outcomes of ALK+ ALCLs. Our data reveal a dynamic chain relation that NPM-ALK regulates p53 protein expression and subsequent apoptosis cascade. They also suggest that inhibition of NPM-ALK by the tyrosine inhibitor herbimycin A reactivate the p53 pathway resulting in inhibition of cellular growth and induction of apoptosis in ALK+ ALCL cells.

ACKNOWLEDGEMENTS

We thank Prof. GU Long Jun (Department of Hematology, Xinghua Hospital affiliated to Shanghai Jiao Tong University, Shanghai) and Prof. HOU Jian (Department of Hematology, Changzheng Hospital affiliated to the Second Military Medical University, Shanghai) for kindly providing Karpas299 and SH-1 cell lines; Prof. HUANG Wei Yi (Faculty of Life Sciences of Nanjing Agricultural College, Nanjing) for herbimycin A drug.

REFERENCES

19. Turturro F, Frist AY, Arnold MD, et al. Biochemical differences between SUDHL-1 and KARPAS 299 cells derived from t(2;5)-positive anaplastic large cell lymphoma are responsible for the different sensitivity to the antiproliferative effect of p27(Kip1). Oncogene, 2001; 20, 4466-75.