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ORIGINAL ARTICLE

Clinical and prognostic importance of XIAP and USP8 in advanced stages of non-small cell lung cancer

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Summary

Purpose: *To investigate the relationship of the apoptosis regulators X-linked inhibitor of apoptosis (XIAP) and ubiquitin specific protease 8 (USP8) with clinical parameters, survival and response to chemotherapy in patients with advanced stages of non-small cell lung cancer (NSCLC).*

Methods: *The study included 34 NSCLC patients (28 females, 6 males) and 44 healthy individuals (17 males, 27 females) as a control group. XIAP and USP8 levels were determined by ELISA.*

Results: *The median serum XIAP level of the patients and the control group showed no significant difference. USP8 level was higher in patients than in controls (p<0.0001). In univariate analysis, there was no significant relationship between XIAP and USP8 serum levels and age, sex, performance status, weight loss, stage of disease, histopatological type and response to chemotherapy. Response to chemotherapy did not differ between the high and low XIAP and USP8 groups . There was no significant difference in progression-free survival (PFS) (p=0.432 and p=0.50, respectively) and overall survival (OS) (p=0.989 and p=0.90, respectively) between the low and high XIAP and USP8 groups.*

Conclusion: *No relationship was found in serum XIAP and USP8 levels with clinical parameters, response to chemotherapy, PFS and OS in patients with advanced stages of NSCLC.*

Key words: *apoptosis, non-small cell lung cancer, protein degradation, USP8, XIAP*

Introduction

Molecular pathways that cause apoptosis are controlled by activation or inhibition of the intracellular cystein proteases, the caspases. The activity of a caspase is inhibited by the inhibitor of apoptosis proteins (IAPs) family. This family has 8 members: NAIP, CIAP1, CIAP2, XIAP, survivin, apollon, livin and ILP-2 [1]. The most studied member of the IAP family is XIAP. XIAP (X-linked inhibitor of apoptosis) does not only inhibit the effectors caspase-3 and caspase-7, but also inhibits the initiator caspase-9. XIAP is a more potent caspase inhibitor than other members of the IAP family in *in vitro* studies [2]. IAPs block apoptotic cell death by inhibiting the activation of specific caspases. This mechanism includes preventing the activation of procaspase-9 and -8, as well as inhibiting caspase-9, -3, -7 and the proteasomal degradation of caspase-9, -3, -7 via ubiquitination [3-5]. However, some of the IAP family members including XIAP can start ubiquitination and proteasomal degradation by binding to endogenous IAPs antagonists (SMAC, HtrA2, ARTS and XAF1), thereby impeding the binding of IAPs to caspases [6-8].

Resistance to apoptosis, chemotherapy and radiotherapy in cancer cells has been linked to the overexpression of survivin and XIAP [9-13]. In malignancies with XIAP and survivin overexpression the prognosis is worse [9-20]. Furthermore, downregulation of XIAP by silencing (si)RNA or

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antisense oligonucleotides (ASOs) enhances chemosensitivity of several tumor cell types [21-23].

The ubiquitin proteasome system (UPS) is an important system in the lysosomal protein degradation pathway [24]. Conjugation of ubiquitin to protein substrates involves multistep processes. Ubiquitin binds to the lysine residues of its substrate by the E1 conjugation enzyme. This conjugation is mediated by ubiquitin conjugases (E2) and ubiquitin ligases (E3) [25]. The covalent binding of target substrates to multiubiquitin molecules by multicatalytic proteosome complexes generally result in protein degradation [26]. Mono- and polyubiquitinations can be reversed by deubuiquitination enzymes (DUBs), which prevent target protein degradation. It is estimated that approximately 95 DUBs are encoded in the human genome [27].

Overexpression of epidermal growth factor receptor (EGFR), a tyrosine kinase receptor, has been discussed in most of the epithelial cancers including lung cancer [28,29]. One of the mechanisms that regulate EGFR expression is ubiquitination. Ubiquitin binds to EGFR and leads to its degradation. Ubiquitin specific protease 8 (USP8) is a de-ubiquitinase and a member of the ubiquitin-specific protease family, regulating the ubiquitination and degradation of EGFR [29-32].

In light of these data, an increasing amount of attention has been paid to suppressing XIAP and USP8 with small molecule inhibitors in the treatment of cancer.

In this research, we aimed to investigate the relationship of serum XIAP and USP8 levels with clinical parameters, as well as examining their effects on survival and response to chemotherapy in patients with advanced stages of NSCLC.

Methods

Patients

The study included 34 locally advanced and metastatic NSCLC patients (28 females,6 males) and 44 healthy individuals (17 males,27 females) as a control group. The median age of the patients was 62.5 years (range 43-87) and 32 years (range 22-52) for the control group.

Methods

The local ethics committee approved this study. Patients and controls were informed about the study and signed a written informed consent, according to the Declaration of Helsinki. Peripheral blood (5 ml) were collected via venopuncture from the 34 patients and the 44 controls. Specimens were collected from patients before chemotherapy and radiotherapy and were centrifuged at 1400 x *g* for 15 min. Serum samples were aliquoted in Eppendorf tubes, and frozen at -86 ^{O}C until use.

ELISA

Human XIAP and USP8 (Usen Life Inc., Wuhan, China) levels were determined by ELISA. Quantitative colorimetric sandwich ELISA was performed according to the manufacturer's recommendations. One hundred microliters of serum sample, depending on the last batch, were added to the wells coated with the capturing antibodies. Dublicate of wells per samples were used. After an incubation period of 2 h at ambient temperature, the wells were washed using a plate washer (Biotek EL.405,USA). Conjugate antibodies and substrate were added and the plates were read at 450 nm using a plate reader (BioTek Synergy HT,USA).

Statistics

Statistical analysis was performed using the SPSS software programme version 15.0. Median XIAP and USP8 levels were taken as cut-off points for statistical analysis.

Low and high XIAP and USP8 groups were compared with the chi-square test according to age, sex, stage, histological type, performance status, weight loss and response to chemotherapy. PFS and OS were estimated with the Kaplan-Meier method and the survival rates were compared with the log rank test. In univariate analyses $p<0.05$ was considered significant. Multivariate analysis was not performed.

Results

Serum XIAP and USP8 levels and clinical parameters

The median age of the patients and controls was 62.5 and 32 years respectively. Seventeen of the 34 (5.9%) patients had stage IV NSCLC, 15 (50%) stage IIIB and 2 (44.1%) stage IIIA. Characteristics of the patients are summarized in Table 1.

The median serum XIAP levels of patients and controls were the same (0.51 ng/ml; range 0.17-2.36). On the other hand, the median USP8 level was higher in patients compared with the control group (0.96 ng/ml; range 0.27-2.56 and 0.495ng/ml; 0.28-5.30, respectively; $p<0.0001$). In univariate analysis, there was no significant relationship of XIAP and USP8 serum levels with age, sex, performance status, weight loss, stage of disease, histopatological type and response to chemotherapy (Table 2).

Relationship of XIAP and USP8 levels and survival

The median follow-up of patients was 10 months (range 1.15-45.1). During the follow-up

Table 1. Patient characterictics

Characteristics	*N (%)*
Age (years)	
≤65	22 (64.7)
>65	12 (35.3)
Gender	
Male	28 (82.4)
Female	6 (17.6)
ECOG performance status	
0-1	21 (61.8)
≥2	13 (38.2)
Weight loss	
Yes	5 (14.7)
No	29 (85.3)
Histology	
Squamous	21 (61.8)
Adenocarcinoma	10 (29.4)
Unknown	3 (8.8)
Stage	
IIIA	2 (5.9)
IIIB	15 (44.4)
IV	17 (50)
Chemotherapy	
Neoadjuvant	6 (17.6)
Palliative	28 (82.4)
Surgery	
Thorasic surgery	6 (17.6)
Cranial metastasectomy	3 (8.8)
Radiotherapy	
Thorax curative	5 (14.7)
Thorax palliative	5 (14.7)
Cranial	8 (23.5)

period, 31 patients died and 3 were alive. Six of the 34 patients (17.6%) had received neoadjuvant chemotherapy before being operated. In 4 of these patients, metastasis developed during follow-up. In 3 of these patients, the XIAP levels were high, while in 1 of them the USP8 level was elevated. There was no significant relationship between the levels of XIAP and USP8 and the time taken for the development of metastasis (log rank p=0.083, p=0.182, respectively). Palliative chemotherapy was given to 28 of the 34 patients (82.4%). Response to chemotherapy between the high and low XIAP USP8 groups did not differ.

Regarding PFS, there was no difference between the low and high XIAP and USP8 groups. The median PFS was 3.29 months (95% CI 0-8.83) in the high XIAP group, 3.35 months (95% CI 3.1-3.6) in the low XIAP group, 3.45 months (95% CI 0-7.7) in the high USP8 group and 3.35 months (95% CI 0.9-1.58) in the low USP8 group (p=0.432 and p=0.50, respectively) (Figure 1A, 1B).

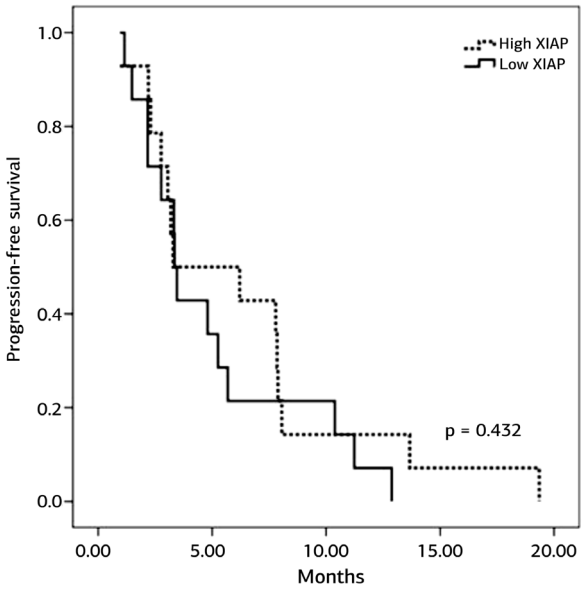

Figure 1A. Kaplan-Meier curves for progression-free survival in relation to serum XIAP levels.

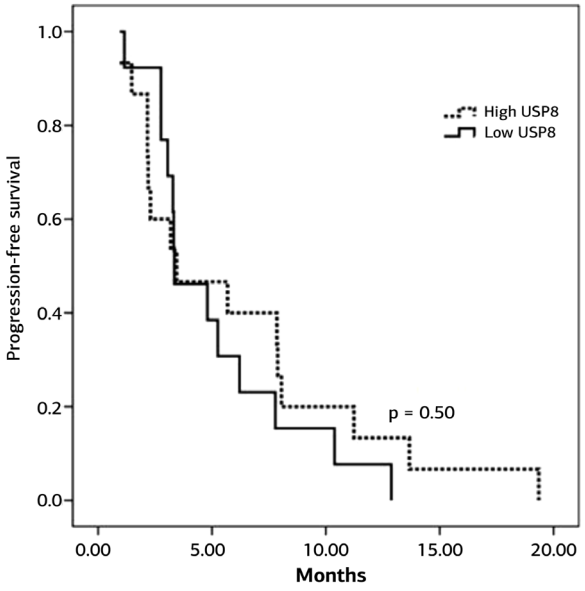

Figure 1B. Kaplan-Meier curves for progression-free survival in relation to serum USP8 levels.

No effect of high XIAP and USP8 levels on prognosis was established. There was no significant difference in OS between the high and low groups. Median OS was 10 months (95% CI 5.76-14.24) and 10.87 months (95% CI 2.75-19) for the high and low XIAP groups, respectively (p=0.989), and 10.87 months (95% CI 7.29-14.45) and 10.48 months (95% CI 4-16.9) for the high and low USP8 groups, respectively (p=0.90, respectively) (Figure 2A, 2B).

Table 2. Relationship between clinical parameters and serum XIAP and USP8 levels

Parameters	*High XIAP*[1]	*Low XIAP*	*p-value*	*High USP8*[2]	*Low USP8*	*p-value*
Age (years)						
≤65	11	11		9	13	
>65	6	6	1.0	8	4	0.151
Gender						
Male	13	15		14	14	
Female	4	2	0.368	3	3	1.0
ECOG performance status						
0-1	10	11		11	10	
≥2	7	6	0.724	6	7	0.724
Weight loss[3]						
Yes	2	3		2	3	
No	15	14	0.628	15	14	0.628
Histology						
Squamous	9	12		10	11	
Adenocarcinoma	6	4		5	5	
Unknown	2	1	0.55	2	1	0.827
Stage[4]						
IIIA	0	2		1	1	
IIIB	8	7		8	7	
IV	9	8	0.346	8	9	0.939
Response to chemotherapy						
Neoadjuvant						
PR	3	2		2	3	
SD	1	0	0.439	0	1	0.439
Palliative						
PR	7	3		6	4	
SD	2	3		3	2	
PD	6	7	0.418	7	6	0.948

[1]Cutoff point at the median level 0.51 ng/ml
[2]Cutoff point at the median level 0.96 ng/ml
[3]More than 10% weight loss in the last 6 months
[4]According to AJCC 6th edition
PR: partial remission, SD: stable disease, PD: progressive disease

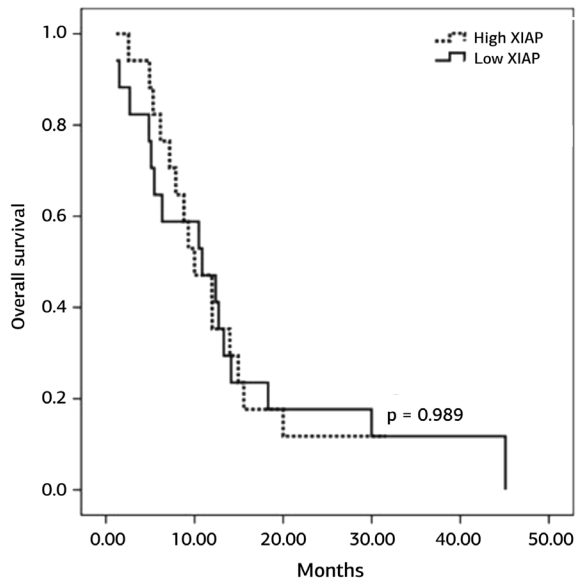

Figure 2A. Kaplan-Meier curves for overall survival in relation to serum XIAP levels

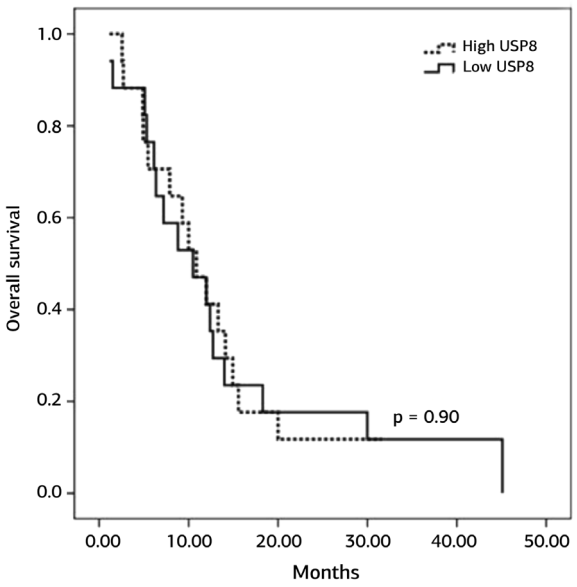

Figure 2B. Kaplan-Meier curves for overall survival in relation to serum USP8 levels.

Discussion

In this study the effects of serum XIAP and USP8 levels on clinical parameters and survival were analyzed. In the study of Krepela and colleagues, survivin expression was found to be significantly higher in NSCLC tumor tissue than in normal lung tissue, while XIAP expression was found to be the same in both NSCLC and normal lung tissue [15]. Hoffmann and his colleagues reported that XIAP expression was higher in normal lung than NSCLC tissue [40-45]. In our study, we did not find significant difference in serum XIAP levels between patients with NSCLC and the healthy control group. This could have been due to the difference in the median age between patients and the healthy group.

In previous studies, it was reported that overexpression of the IAP family was associated with poor prognosis in acute myeloid leukemia, lymphoma, colorectal cancer, renal cell cancer, gastric cancer and NSCLC [18,33-38]. However, no relationship between XIAP levels and survival could be demonstrated in colon, cervix and bladder cancers [10,16,17].

In two different studies, Carlos and colleagues did not observe a relationship between IAP1, IAP2 and XIAP overexpression and age, sex, grade, histology and response to chemotherapy in patients with advanced stages of NSCLC. Moreover, they found that the IAP1, IAP2 and XIAP levels did not affect time to progression and overall survival [16]. In another study by Carlos and colleagues on patients with NSCLC who had undergone radical resection, the survival of patients with XIAP overexpression was found to be longer. There was no correlation between XIAP expression and the apoptotic index, but there was an inverse correlation between XIAP and Ki-67 and the mitotic index [17]. We also found similar results in that there was no relationship of serum XIAP levels with clinical parameters, response to chemotherapy and prognosis.

Although not proven in clinical studies, in cell line studies using small molecule inhibitors or antisense oligonucleotides of XIAP and IAPS, apoptosis was induced and chemosensitivity of tumor cells increased [21,22,41,42].

Dean and colleagues demonstrated that the combination of small molecule XIAP inhibitors with cisplatin and vinorelbine induced apoptosis [23]. Moreover, these inhibitors showed a synergistic effect with the chemotherapeutic agents [23,41,42].

However, some of the members of the IAP family including XIAP can start ubiquitination and proteasomal degradation by binding to IAP antagonists (SMAC/DIABLO, HtrA2, ARTS and XAF1), thereby preventing the binding of IAPs to caspases. Some studies showed that the relative ratio of XIAP to SMAC/DIABLO determined survival [5-8,45-47]. Maybe this is the reason why different results were obtained in the clinical studies. XIAP levels have been measured *in vitro* and in paraffin blocks, as well as in serum samples from cancer patients. However, the results from all these investigations are not sufficient to understand the functions of XIAP and the other members of the IAP family, and their interaction with inhibitory molecules.

Ubiquitin is a major molecule in protein degradation. It also functions as an important mediator in intracellular signaling cascades [48]. Ubiquitination of target proteins can be reversed by DUBs. These enzymes can affect the stability of key oncogenes or can negatively regulate ubiquitin-mediated signaling. Deubiquitinases are important regulators of oncogenes and tumor supressor genes [49-55]. Their overexpression or loss of function can trigger carcinogenesis. In lung, ovarian, breast, prostate and hepatocellular cancers and malignant melanoma, expression of deubiquitinases is increased or decreased [56,56]. In our study, serum USP8 levels were higher in patients with NSCLC than in the healthy control group.

Both the oncogenic and the tumor suppressor functions of deubiquitinases have been defined, but it is difficult to determine their exact course of *in vivo* oncogenic and tumor suppressor functions [57]. In lung cancer and almost all epithelial cancers, tyrosine kinase receptors (TKR) are overexpressed [28,29]. One of the mechanisms that regulate EGFR is ubiquitination. Ubiquitin causes degradation of EGFR by binding to it. USP8 is a deubiquitinase and a member of the ubiquitin-specific protease family, regulating the ubiquitination and degradation of EGFR. However, there are contradictory results with regard to this issue. In one study USP8 was reported to prevent the degradation of EGFR by deubiquitination in endosomes [30]. On the other hand, two other studies demonstrated that UPS8-mediated ubiquitination was needed for EGFR degradation [31,32].

In a study performed with USP8 knock-out mice, the levels of the tyrosine kinases EGFR, c-met and ErbB3 were significantly decreased. The results of this report questioned the proliferative role of USP8 [58].

In our study, serum USP8 levels were high in patients with NSCLC. However, no effects of USP8

levels on clinical parameters, response to chemotherapy, PFS and OS could be shown.

In conclusion, we observed no relationship of serum XIAP and USP8 levels with clinical parameters, response to chemotherapy, PFS and OS in patients with advanced stages of NSCLC. The low number of patients in our study could be one of the reasons for these negative results. Furthermore, the *in vivo* roles of both XIAP and USP8 in carcinogenesis are quite complex. Further studies are needed to define the functions of both molecules, their interactions with other molecules in the apoptotic pathway, and their association with prognosis and survival.

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