Table 5 Factors predicting peritoneal dissemination in recurrent ovarian cancer

Variable	Number	Dissemination, *n* (%)	*P* value
Cytoreduction			
Secondary	21	4 (19.0)	0.608
Tertiary	4	1 (25.0)	
Quaternary	2	1 (50.0)	
Histology			
Serous	20	6 (30.0)	0.155
Other	7	0 (0.0)	
Grade			
1/2	9	1 (11.1)	0.381
3	18	5 (27.8)	
TFI (months)			
<12	12	6 (50.0)	0.0031
≧12	15	0 (0.0)	
CA 125 (U/ml)			
<100	19	4 (21.1)	0.773
≧100	8	2 (25.0)	
Number of recurrent lesions			
1	19	2 (10.5)	0.136
2	8	4 (50.0)	
Size of largest tumour (mm)			
<30	14	4 (28.6)	0.648
≧30	13	2 (15.4)	
Ascites at operation			
No	24	3 (12.5)	0.0068
Yes	3	3 (100.0)	

detection of asymptomatic recurrence by routine surveillance testing is associated with a high likelihood of optimal secondary cytoreductive surgery and extended overall survival [7].

During the course of recurrent treatment, several strategies can be used, namely: chemotherapy, cytoreductive surgery and palliative irradiation. To avoid an accumulation of adverse effects of systemic cytotoxic agents (e.g. peripheral neuropathy, myelosuppression), we considered that it would be preferable to offer site-specific therapy for patients with recurrence when possible. Individualized approaches are also essential for a good quality of life and long survival of patients. For instance, patients who show symptoms or massive ascites need immediate treatment. On the other hand, in an asymptomatic patient who shows only rising serum CA 125 levels and negative or equivocal CT scans, a PET scan is strongly recommended. The presence, region and uptake pattern of FDG should be fully reviewed in order to make a decision. The management plan for the patient should then be discussed and confirmed. In some patients, close observation is recommended if there is no distinct accumulation on the PET scan despite elevated serum CA 125. Systemic chemotherapy is given for patients who indicate multiple or diffuse FDG uptake patterns. Otherwise, cytoreductive surgery is strongly considered for patients whose FDG uptake patterns are localized.

The sensitivity and specificity of CA 125 in recurrent ovarian cancer diagnosis are reported to be 57.6 – 92.1 % and 71.9 – 96.7 %, respectively [8–10]. And the sensitivity and specificity of PET/CT are 93.3 – 97.4 % and 80 – 100 %, respectively [11, 12]. A recent meta-analysis of 29 studies demonstrated that PET/CT has high sensitivity (89 %) and specificity (90 %) [13]. Some studies have shown that management plans tend to change after examination of PET scans [14, 15]. Fulham et al. found that the management plan in 58.9 % of patients changed based on PET scan findings. In that study, 38.9 % of the patients finally underwent cytoreductive surgery [16]. In our study, the results of 58.4 % of the PET scans led to a change in management of the patient. The proportions of patients who were treated surgically increased from 13.5 % before PET to 39.3 % after PET. The use of PET/CT in patients with elevated CA 125 levels would lead to more patients being appropriately offered either chemotherapy or surgery.

Patients with localized FDG uptake patterns are the best candidates for surgical therapy. Patients with a TFI of more than 6 months were more likely to have higher localized FDG uptake. Of patients who had a positive PET scan, 78 % exhibited a localized pattern if their TFI was more than 12 months. Whether there is an association between TFI after second recurrence and outcome of the recurrent treatment is still a matter of debate. And there are very limited data published on tertiary surgical cytoreduction [17]. Notably, serum CA 125 levels have been found to be lower in patients with localized FDG uptake than in those with multiple or diffuse patterns. Palomar et al. consider that a PET scan is indicated when the CA 125 level is above 18 U/ml [18]. We observed that the proportion of patients with a positive PET scan was significantly higher (29/35, 82.9 %) when the CA 125 level exceeded 20 U/ml, compared to those with a level lower than 20 U/ml (4/27, 14.8 %; $P<0.01$; unpublished data). A localized FDG uptake pattern would probably be obtained when (a) the TFI is ≥6 months and (b) there is a successive elevation of serum CA 125 above 20 – 30 U/ml. In these patients, cytoreductive surgery should be considered.

A recent meta-analysis has revealed that the optimal level of cytoreduction after secondary cytoreductive surgery is 70.3 % (range, 22.2 – 100 %) [3], although the definition of optimal cytoreduction varied (from <2.5 cm to no gross disease). The proportion of patients with complete resection was lower (52.2 %, range 9.4 – 100 %). Multivariate analysis revealed that the proportion of patients with complete cytoreduction and the year of publication were significant predictors of survival. Each 10 % increase in the proportion of patients with complete cytoreductive surgery was associated with a 3-month increase in median cohort survival time. However, disease-free interval and survival were not significantly associated [3]. For tertiary

cytoreduction, only residual disease after surgery retained prognostic significance [17]. Consequently, the selection of appropriate candidates is crucial for optimal cytoreduction. The results of our study suggest that the FDG uptake pattern is useful for selecting patients who are suitable for site-specific treatment. In this study, the rate of complete resection was as high as 91.4 %, and median tumour size was small (30 mm). Moreover, there were no severe complications or perioperative mortality. It is reasonable to suppose that we were able to choose optimal candidates using PET scanning and to perform cytoreduction earlier than in previous studies. This may have led to a high complete resection rate and low morbidity.

FDG PET scanning produces functional images that reflect increased rates of glucose metabolism in tumour, and it has many pitfalls in clinical use. Many papers in oncology have reported that PET scanning is of limited use in the detection of malignant tumours less than 1 cm in size. In the current study, six patients with localized macroscopic recurrence were also found to have miliary or multiple small lesions (<5 mm). None of these lesions was detected by PET. All patients in whom complete resection was not possible had miliary disease. Size limitations in performing PET scans must be born in mind, since implants <10 mm are inconsistently identified owing to low concentrations and limited spatial resolution [19]. Miliary peritoneal dissemination was significantly associated with a TFI of <12 months and ascites on cytoreduction. Consequently, even if a PET scan indicates localized FDG uptake, whether cytoreductive surgery should be performed in patients with a TFI of <12 months needs careful consideration.

In summary, PET scanning is helpful in optimizing the management plan in patients with recurrent ovarian cancer and in aiding in the selection of appropriate candidates for attempted surgical resection.

Conflicts of interest None.

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RESEARCH ARTICLE

An Exploratory Study of Japanese Fathers' Knowledge of and Attitudes towards HPV and HPV Vaccination: Does Marital Status Matter?

Sharon Janet Bruce Hanley[1,2]*, Eiji Yoshioka[2,4], Yoshiya Ito[3], Ryo Konno[5], Yuri Sasaki[5], Reiko Kishi[2,6], Noriaki Sakuragi[1]

Abstract

Background: No studies on male attitudes towards HPV and HPV vaccination have been conducted in Japan, and little is known globally whether attitudes of single fathers differ to those living with a female partner. This exploratory study assessed whether Japanese fathers were likely to have their daughter vaccinated against HPV in a publically funded program and whether any differences existed regarding attitudes and knowledge about HPV according to marital status. Materials and Methods: Subjects were 27 fathers (16 single; 11 married) who took part in a study on HPV vaccine acceptability aimed at primary caregivers of girls aged 11-14 yrs in three Japanese cities between July and December 2010. Results: Knowledge about HPV was extremely poor (mean score out of 13 being 2.74±3.22) with only one (3.7%) participant believing he had been infected with HPV and most (81.4%) believing they had no or low future risk. No difference existed regarding knowledge or awareness of HPV according to marital status. Concerning perceived risk for daughters, single fathers were significantly more likely to believe their daughter was at risk for both HPV (87.5% versus 36.4%; p=0.01) and cervical cancer (75.0% versus 27.3%; p=0.02). Acceptability of free HPV vaccination was high at 92% with no difference according to marital status, however single fathers were significantly more likely (p=0.01) to pay when vaccination came at a cost. Concerns specific to single fathers included explaining the sexual nature of HPV and taking a daughter to a gynecologist to be vaccinated. Conclusions: Knowledge about HPV among Japanese fathers is poor, but HPV vaccine acceptability is high and does not differ by marital status. Providing sexual health education in schools that addresses lack of knowledge about HPV as well as information preferences expressed by single fathers, may not only increase HPV vaccine acceptance, but also actively involve men in cervical cancer prevention strategies. However, further large-scale quantitative studies are needed.

Keywords: HPV vaccine - knowledge - attitudes - fathers - daughters

Asian Pac J Cancer Prev, **15 (4)**, 1837-1843

Introduction

Persistent infection with an oncogenic human papillomavirus (HPV) is responsible for almost all cases of cervical cancer (de Sanjose et al., 2010; Li et al., 2011), and to a lesser extent cancer of the vagina, vulva, penis, anus and oropharynx (Kreimer et al., 2005; De Vuyst et al., 2009; Ferlay et al., 2010; de Martel et al., 2012). While both men and women are equally responsible for spreading the virus, the burden of HPV related disease is considerably higher in women, with around 570,000 HPV related cancer cases annually in women compared to only 34,000 in men (Arbyn et al., 2012). As a result, much of the research on HPV awareness and knowledge has focused primarily on women (Waller et al., 2003; Oh et al., 2010; Gunasekaran et al., 2012; Hanley et al., 2012). Data on men's attitudes and knowledge is less common. However, one study demonstrated a five-fold increased risk of cervical cancer in women who had a male partner infected with penile HPV (Bosch et al., 1996). Furthermore, extramarital partners and number of lifetime partners in males is also associated with HPV detection and increased risk for cervical cancer in female partners (Zunzunegui et al., 1986; Burk et al., 1996). The World Health Organization (WHO) position paper on HPV vaccines states that these vaccines should be introduced

*[1]Department of Reproductive Endocrinology and Oncology, [2]Department of Public Health, Graduate School of Medicine, Hokkaido University, Sapporo, [6]Center for Environmental and Health Sciences, Hokkaido University, Sapporo, [3]Department of Clinical Medicine, Faculty of Nursing, Japanese Red Cross Hokkaido Red Cross College of Nursing, Kitami, [4]Department of Health Science, Faculty of Medicine, Asahikawa Medical University, Asahikawa, [5]Department of Obstetrics and Gynecology, Faculty of Medicine, Jichi Medical University, Saitama Medical Center, Saitama, Japan *For correspondence: sjbh1810@med.hokudai.ac.jp*

as part of a coordinated prevention strategy for cervical cancer and other HPV-related diseases. It also states that the strategy should include education on risk-reducing behavior (World Health, 2009). Thus, given that the sexually transmitted nature of HPV implies recognition that sexual behavior in both men and women is a risk factor for cervical cancer, and, since most HPV infections in men are asymptomatic and screening of this population is not standard clinical practice, better outcomes for cervical cancer prevention programs may be achieved if men were actively included in HPV education and awareness campaigns.

Two highly effective prophylactic HPV vaccines have been developed that contain antigens against HPV types 16 and 18, responsible for around 70% of cervical cancer cases worldwide (Ault and Future, 2007; Paavonen et al., 2009). One vaccine, the quadrivalent vaccine, also contains antigens against HPV types 6 and 11 and affords high protection against genital warts (Garland et al., 2007). Because pre/adolescent girls are the primary target of HPV vaccination programs, understanding parental attitudes to HPV vaccination is essential. In Japan, free HPV vaccination became available from 2011 for girls aged 12-16yrs (Infectious Disease Surveillance Center, Immunization Schedule, 2011). While mothers are the primary decision-makers for children's healthcare in Japan, divorce or illness may result in fathers being the sole-caregiver. However, one US study on parental attitudes towards a herpes simplex virus type 2 (HSV-2) vaccine showed fathers were significantly more likely than mothers to refuse a vaccination for a disease that was sexually transmitted (Liddon et al., 2005).

Since no data exists on Japanese men's knowledge of and attitudes towards HPV and HPV vaccination and little is known globally about whether attitudes of single fathers differ to those living with a female partner, the aim of this exploratory study was to assess to what extent Japanese fathers of adolescent girls were likely to have their daughter vaccinated against HPV in a publically funded program and to investigate whether any differences exist regarding attitudes and knowledge about HPV, according to marital status.

Materials and Methods

Participants and procedure

Participants were 27 fathers who took part in a study of primary caregivers with adolescent daughters in school years 5-8 (ages 11-14yr) in two medium-sized (population 100,000) Japanese cities and one large city (population 2 million) in Northern Japan between July and December 2010. Details about the procedure have been reported elsewhere (Hanley et al., 2012), but in brief, a self-administered questionnaire, a stamp addressed envelope and a letter explaining the purpose of the study addressed to the primary caregiver were distributed through the schools and returned to the main investigator by post. The study was approved by the Ethics Review Board for Epidemiological Studies at Hokkaido University Graduate School of Medicine. Since the survey was both voluntary and anonymous, completing the questionnaire was taken as consent to participate in the study.

Survey instrument and measures

A 103-item survey instrument was developed based on previous research on vaccine acceptability and adapted for a Japanese population (Dempsey et al., 2006; Fazekas et al., 2008). It assessed parental attitudes towards routine childhood vaccinations, socio-demographic factors (age, number of children, marital status, educational background, annual household income and disposable monthly income), knowledge about and attitudes towards cervical cancer, HPV and the HPV vaccination, and willingness to pay for HPV vaccine. To ensure primary caregivers had some understanding of the HPV vaccine, the following information was included: 'There are over 100 types of HPV that infect humans. Some types cause cancer (oncogenic HPV) and other types that do not. The oncogenic types of HPV that are responsible for causing cervical cancer are mainly transmitted by sexual intercourse. Two new vaccines (HPV vaccine) have been developed that prevent infection with the two most oncogenic HPV types. It is estimated that these vaccines can prevent up to 70% of all cervical cancers. One vaccine also protects against the two most common HPV types that cause genital warts. It is estimated that this vaccines can prevent around 95% of new cases of genital warts'.

HPV vaccine acceptability

Since national funding was not available for the HPV vaccine at the time of the study, vaccine acceptability was assessed by examining intentions to vaccinate if the vaccine was free, or if participants had to pay the minimum recommended price of ¥40,000 (around US$400). Intention to vaccinate when free was assessed by the question: 'If your daughters could have the HPV vaccine for free, how likely would you be to have her vaccinated'. Responses were on a 5-point scale ('very unlikely', 'unlikely', 'not sure', 'likely', 'very likely'). Willingness to pay for HPV vaccination was measured by the question, 'What is the most you would be willing to pay out of pocket to have your adolescent daughter vaccinated against HPV'. Responses were on an 8-point scale: 'Nothing', '¥100-1,999', '¥2,000-4,999', '¥5,000-9,999', '¥10,000-19,999', '¥20,000-29,999', '¥30,000-39,999' and '¥40,000 or more'.

Knowledge and attitudes regarding HPV and HPV vaccine

To measure knowledge about HPV, we used 13 questions adapted from previous research (Dempsey et al., 2006; Fazekas et al., 2008). Questions included ten true-false statements and three composite questions about symptoms of HPV, consequences of untreated HPV and risk for HPV infection.

To assess participants' attitudes toward HPV and HPV vaccination, questions based on five concepts from the Health Belief Model (HBM) (Maiman et al., 1977) perceived susceptibility to HPV infection; perceived severity of HPV infection; perceived benefits of HPV vaccination; perceived barriers to HPV vaccination and cues to action for HPV vaccination, such as recommendation from a doctor or local health board,

were used. The HBM postulates that health behavior is determined by personal beliefs or perceptions about a disease, as well as the strategies available to reduce its occurrence, and has been used in several studies on HPV vaccine acceptability (Dempsey et al., 2006; Fazekas et al., 2008; Marlow et al., 2009). Perceived benefits and barriers were assessed with questions on vaccine efficacy and safety.

Statistical analysis

Data were analyzed using IBM SPSS Statistics Version 20.0 (SPSS Inc., Chicago, USA). Fathers indicating 'likely' or 'very likely' to have their daughter vaccinated were classified as 'acceptors', and those answering 'very unlikely', unlikely' or 'not sure' were classified as 'non-acceptors'. Bivariate analyses using Pearson's chi-squared and Fisher's exact test for categorical variables and Mann-Whitney U test for continuous variables were used to compare differences in attitudes and knowledge about HPV and HPV vaccine between fathers who were married or cohabiting (hereafter referred to as 'married') and those who were divorced, widowed or separated (hereafter referred to as 'single'). Due to the small number of non-acceptors and small sample size, multivariable logistic regression was not performed. Statistical significance was defined as a 2-tailed p-value of <0.05.

Results

Socio-demographics

Background characteristics of participants are shown in Table 1. The majority of participants were aged between 40 and 49 yrs (mean age 44.85 yrs±6.13), single (59.3%), did not have a university degree (55.5%) and had a disposable monthly income of <30,000 yen ($300). There were no statistically significant differences in socio-economic factors, including marital status, between those fathers willing to have their daughter vaccinated and those who were not.

Knowledge about HPV

While 37% of fathers stated they had heard of HPV, accurate knowledge about HPV infection was poor (mean score out of a possible 13 was 2.74±3.22), and no significant difference was found with regards to marital status (Table 3). As shown in Table 2, only 18.5% of participants could correctly name the risk factors for HPV infection and only 7.4% knew the symptoms. For most questions, the most common answer was 'don't know' (data not shown). Furthermore, even though participants were actually told in the questionnaire that HPV causes cancer and a vaccine had been developed that prevent infection with the two most oncogenic types that cause cervical cancers, only 37.7% correctly answered that HPV was indeed the cause of cervical or any other cancer. However, despite overall knowledge being poor, 66.7% of participants did indicate that they would like more information on HPV (Table 3).

Awareness of and attitudes towards HPV and HPV vaccination

Only one (3.7%) participant believed he had been previously infected with HPV and most (81.4%) believed they had absolutely no or low future risk of infection. There were no significant differences according to marital status (Table 3). However, with regards to their adolescent daughter's future risk for HPV infection, single fathers were significantly more likely to admit their daughters had a medium to high future risk compared to fathers living with a female partner (87.5% and 36.4%, respectively; p=0.01). Similarly, they were also more likely to believe their daughters was at risk for cervical cancer (75.0% and 27.3%, respectively; p=0.02).

While only half (51.9%) had heard of the HPV vaccine, 92.5% of father's were willing to vaccinate their daughter if offered for free. Although there was no statistical difference according to marital status, all (100%) single fathers stated they would have their daughters vaccinated in a publically funded HPV vaccination program. Single fathers were also significantly more likely to be willing to pay more for the vaccine (p=0.01), when vaccination came at a cost (Table 3). While most fathers (63.3%) stated they would use the internet as the first place to go for more

Table 1. Selected Characteristics of the Sample Population and HPV Vaccine Acceptance

Characteristic	Overall N (%)	Acceptors N (%)	Non-Acceptors N (%)	p-value
Age (yr)				
29-39	5 (18.5)	4 (80.0)	1 (20.0)	
40-49	16 (59.3)	15 (93.8)	1 (7.2)	
>50	6 (22.2)	6 (100.0)	0 (0.0)	0.26
Marital Status				
Married/cohabiting	11 (40.7)	9 (81.8)	2 (18.2)	
Separated/divorced/widowed	16 (59.3)	16 (100.0)	0 (0.0)	0.16
Education				
Less than university	15 (55.5)	14 (93.3)	1 (6.7)	
University or more	12 (44.5)	11 (91.7)	1 (8.3)	1.00
Annual Household Income (yen)a				
<5 million	12 (44.4)	12 (100.0)	0 (0.0)	
5-<7 million	11 (40.7)	9 (81.8)	2 (18.2)	
>7 million	4 (14.9)	4 (100.0)	0 (0.0)	0.17
Monthly Disposable Income (yen)				
<30,000	16 (59.3)	14 (87.5)	2 (12.5)	
>30,000	11 (40.7)	11 (100.0)	0 (0.0)	0.50

Table 2. Fathers' Knowledge about HPV

Statement[a]	Correct Response Overall n (%)
HPV is the virus that causes herpes (F)	4 (14.8)
Genital warts are caused by some types of HPV (T)	7 (25.5)
HPV is the virus that causes cervical cancer (T)	10 (37.7)
Pap smears prevent disease caused by HPV (T)	13 (48.1)
If a women has a normal Pap smear, she doesn't have HPV (F)	8 (29.6)
Changes in a Pap smear may indicate a woman has HPV (T)	11 (40.7)
Genital warts are caused by the herpes virus (F)	3 (11.1)
HPV can cause cancer (T)	10 (37.7)
Pap smears will almost always detect HPV (F)	5 (18.5)
HPV can be passed from mother to child during childbirth (T)	3 (11.1)
Risk factors for HPV infection[b]	5 (18.5)
Symptoms of HPV infection[c]	2 (7.4)
Consequences of untreated HPV infection[d]	9 (33.3)

[a]T:True, F:False, [b]Correct if respondent marked two of three correct responses (sex before age 16, many sexual partners, or partner with many sexual partners), [c]Correct if respondent marked two of three correct responses (warts that sometimes itch or bleed, warty growths, or no symptoms), [d]Correct if respondent marked four of six correct responses (cancer, dysplasia, unable to give birth, warts, no consequences, or death)

Table 3. Fathers' Awareness of and Attitudes Towards HPV and HPV Vaccine According To Marital Status

	Overall Mean (SD)	Marital status Married Mean (SD)	Single Mean (SD)	p value
HPV knowledge score	2.74 (3.22)	3.00 (3.31)	2.56 (3.31)	0.74
	N(%)	N(%)	N(%)	
Heard of HPV				
Yes	10 (37.0)	4 (36.4)	6 (37.5)	
No	15 (55.6)	6 (54.5)	9 (56.2)	
Don't Know	2 (9.4)	1 (9.1)	1 (6.3)	1.00
Believe may have had a previous HPV infection				
Yes	1 (3.7)	1 (9.1)	0 (0.0)	
No	14 (51.9)	7 (63.6)	7 (43.8)	
Don't Know	12 (44.4)	3 (27.3)	9 (56.3)	0.10
Beliefs about future risk of HPV Infection				
Absolutely no risk	11 (40.7)	5 (45.5)	6 (37.5)	
Low risk	11 (40.7)	5 (45.5)	6 (37.5)	
Medium to high risk	5 (18.5)	1 (9.1)	4 (25.0)	0.21
Perceived threat to one's health from HPV infectionb				
None to low	4 (15.4)	2 (18.2)	2 (13.2)	
Medium to very high	22 (84.6)	9 (81.8)	13 (86.7)	0.74
Would like more information on HPV				
Yes	18 (66.7)	7 (63.6)	11 (68.8)	
No/No preference	9 (33.3)	4 (36.4)	5 (31.3)	1.00
Beliefs about daughter's future risk for HPV infection				
No risk to low risk	9 (33.3)	7 (63.6)	2 (12.5)	
Medium to high risk	18 (66.6)	4 (36.4)	14 (87.5)	0.01**
Beliefs about daughter's future risk for cervical cancer				
No risk to low risk	12 (44.4)	8 (72.2)	4 (25.0)	
Medium to high risk	15 (55.6)	3 (27.3)	12 (75.0)	0.02*
Heard of vaccine to prevent cervical cancer				
Yes	14 (51.9)	5 (45.5)	9 (56.3)	
No	10 (37.0)	5 (45.5)	5 (31.3)	
Don't Know	3 (11.1)	1 (9.0)	2 (12.5)	0.67
Willing to vaccinate if free				
Yes	25 (92.5)	9 (81.8)	16 (100)	
No/ Don't know	2 (7.4)	2 (18.2)	0 (0.0)	0.16

	Overall N (%)	Marital status Married N (%)	Single N (%)	p value
Price willing to pay for vaccination (yen)a				
100-1,999	7 (25.9)	4 (36.4)	3 (18.8)	
2,000-4,999	7 (25.9)	1 (9.1)	6 (37.5)	
5,000-9,999	10 (37.7)	5 (45.5)	5 (31.3)	
10,000-19,999	1 (3.7)	1 (9.1)	0 (0.0)	
20,000-29,999	1 (3.7)	0 (0.0)	1 (6.3)	
30,000-39,999	0 (0.0)	0 (0.0)	0 (0.0)	
>40,000 yen (current price)	1 (3.7)	0 (0.0)	1 (6.3)	0.01**
Preferred age for vaccination (yrs)				
0-9	1 (3.7)	0 (0.0)	1 (6.3)	
10-13	20 (74.1)	9 (81.8)	11 (68.8)	
15-18	6 (22.2)	2 (18.2)	4 (25.4)	0.61
HPV vaccine is effective against HPV				
Yes	21 (77.8)	9(81.8)	12 (75.0)	
No/Don't know	6 (22.2)	2(18.2)	4 (25.0)	1.00
Believe HPV vaccine is safe				
Yes	13 (48.1)	4(36.4)	9(56.2)	
No/Don't know	14 (51.9)	7(63.6)	7(43.8)	0.31
Believe HPV vaccine has serious side effects				
Yes	8 (29.6)	3(27.3)	5 (31.2)	
No/Don't know	19 (70.4)	8(72.2)	11(68.8)	0.82
Desire more information on HPV vaccine				
Yes	18 (66.7)	7 (63.6)	11 (68.8)	
No/No preference	9 (33.3)	4 (38.9)	5 (31.3)	1.00
First place to go for more information				
Internet	17 (63.3)	8 (72.7)	9 (56.3)	
Medical professional	5 (18.5)	1 (9.1)	4 (24.0)	
Book	4 (14.8)	2 (18.2)	1 (12.5)	
Helpline	1 (3.7)	0 (0.0)	1 (6.3)	0.57
Recommendation from a doctor				
Will encourage intention to vaccinate	9 (33.3)	2 (18.2)	7 (43.8)	
Will have no effect on intention to vaccinate	18 (66.7)	9 (81.8)	9 (56.3)	0.23
Recommendation from the local health board				
Will encourage intention to vaccinate	10 (37.7)	1 (9.1)	9 (56.3)	
Have no effect/discourage intention to vaccinate	17 (63.0)	10 (90.9)	7 (43.6)	0.02*

*p<0.05; **p<0.01; a100yen =$1:00

information on the HPV vaccine, more single fathers reported they would consult a health professional (24.0%) compared to married fathers (9.1%). Recommendation from the local health board would also significantly increase acceptance in single fathers (p=0.02). No differences were found regarding perceptions of vaccine efficacy and safety.

Concerns specific to single fathers

At the end of the questionnaire, participants were given the opportunity to write any comments or concerns they had about the vaccine. One single father wrote, "I have a 14yr old daughter, I want her to be vaccinated but I am a man. I can't take her to the gynecologist and she says she is too old for a pediatrician. I wish she could be vaccinated in school". Similarly, another father said, "I have one daughter. She is 13yrs old. I will pay anything so she doesn't get cancer like her mother. But if HPV is transmitted sexually, how can I talk to her about the vaccine? I am a man. I want her school to provide more education about the vaccine". One other father who lost his wife to breast cancer also wrote, "I have 3 daughters, aged 12, 15 and 17yrs. My wife died of cancer. I don't want my girls to get cancer. But I can't afford 15,000 yen per dose to vaccinate all my daughters. Please try and persuade the government to fund the vaccine". Most of the single fathers in this study who were widowed had lost their wife to cancer and while they all wanted to protect their daughters from cervical cancer, they expressed a sense of frustration at the hurdles facing them.

Discussion

No data exists on Japanese men's knowledge of and attitudes towards HPV and HPV vaccination, and little is known globally about whether attitudes of single fathers towards vaccinating their adolescent daughters against HPV differ to those living with a female partner. To address this gap in knowledge we carried out an exploratory study of 27 Japanese fathers with daughters aged 11-14yrs.

Compared to one Honduran study (Perkins et al., 2012) and one German study (Kuznetsov et al., 2012) where 22% and 29% of fathers, respectively, had heard of HPV, awareness in this study was slightly higher at 37%, but considerably lower than a recent Italian study where 77% of fathers had heard of HPV (Pelucchi et al., 2010). Higher education level in the Italian study is suggested to be one of the reasons for the difference. However in both the present study and the Italian paper just over 40% of fathers had a university education, so this difference in awareness may reflect the lack of education about HPV and cervical cancer at all levels of the Japanese education system. One other reason for the difference may be the way in which the HPV vaccine has been promoted in Japan. Unlike in several European countries where the vaccine has been promoted as an 'HPV vaccine against cervical cancer', at the time of the study, only the bivalent vaccine was licensed in Japan and promoted mainly as a "vaccine against cervical cancer". This may also explain why considerably more fathers (52%) were aware of a vaccine to prevent cervical cancer rather than the actual virus (37%) that causes it.

We found that men greatly underestimated their own past and future risk for HPV infection, particularly since 60% of them were single. Similar results have been reported in the US (McPartland et al., 2005) and Europe (Verhoeven et al., 2006). In contrast, one Japanese survey conducted by the Japan Broadcasting Corporation (NHK) found that 11% of Japanese men aged 16-69yrs had more than one sexual partner in the past year and of those 52% were in sexual relationships with several partners simultaneously. Furthermore, 12% of married men stated they had extramarital sex within the past year, with a further 20% declining to answer (NHK, 2002). One Indian study also reported that for women with only one lifetime sexual partner, premarital and, in particular, extramarital relationships in their husband, increased their risk of cervical cancer by up to 6.9-fold (Agarwal et al., 1993). Until now, cervical cancer prevention strategies in Japan have focused solely on women with secondary prevention in the form of screening using cervical cytology, and from 2011 with cytology and high-risk HPV testing for low grade abnormality triage. However, now that primary prevention has become possible with the development of two HPV vaccines, and given that the sexually transmitted nature of HPV implies recognition that sexual behavior in both men and women is a risk factor for cervical cancer, along with the fact that HPV does also cause cancer in men, Japanese men need to be actively involved in future HPV education and awareness campaigns as a strategy for cervical/cancer prevention, and preferable before they reach adulthood.

While no difference existed regarding personal susceptibility to HPV infection and marital status, when it came to perceived risk for participant's adolescent daughter, single fathers were significantly more likely to perceive a risk, and that risk was similar to perceived risk reported by mothers in the same study (Hanley et al., 2012). This suggests that being the parent who shoulders most of the responsibility for their child's healthcare rather than the gender of the parent is a determining factor for admitting a child's susceptibility to a virus that is sexually transmitted or to illness in general, and may also explain why fathers in the study by Liddon et al. were less likely than mothers to accept the HSV-2 vaccine (Liddon et al., 2005).

Despite limited knowledge and awareness about HPV, fathers' acceptance of the HPV vaccine was high (92%) and comparable to that of mothers (Hanley et al., 2012) in the same study. It was also similar to fathers in Honduras (94%) (Perkins et al., 2012) and considerably higher (65%) than the Italian study (Pelucchi et al., 2010), indicating that father's awareness of HPV is not a measure of HPV vaccine acceptance. While all single fathers in both the present and Honduran study indicated they would vaccinate their daughter against HPV, our study did highlight some issues specific to these fathers, such as talking about the sexual nature of HPV or place of vaccination, that need to be addressed. Japan has no school-based childhood vaccination program and there is also no general practitioner (GP), so in most cases

pediatricians are the primary health care provider for children. However, several studies have shown that girls of mothers who attend a gynecologist regularly are more likely to be vaccinated against HPV (Chao et al., 2009; Lefevere et al., 2011; Hanley et al., 2012). Since gynecologists may be more active in recommending (to a mother) that a child be vaccinated against a disease they see on a daily basis, single fathers, particularly of older adolescent girls, may missing out on the counseling they need or desire. Providing adequate education about HPV to both sexes in schools would not only help resolve this issue, but also involve men in cervical cancer prevention strategies. In the absence of school-based education, Japanese pediatricians need to be more aware of the potential issues facing single fathers of adolescent girls with regards to HPV vaccination, as well as providing guidance about the ongoing need for cervical screening in future years.

The results of this exploratory study need to be interpreted with a great deal of caution because the sample size was very small and, while socioeconomically diverse, cannot be taken as representative of the general population. Furthermore, we investigated intention to vaccinate which might overestimate actual uptake since external barriers such as having to take time off work (particularly difficult for Japanese men), three times over a six month period may be great. Nevertheless, we have identified some potentially important issues concerning Japanese men's lack of knowledge about HPV, as well as problems unique to single fathers with adolescent daughters regarding HPV vaccination. As a result, we have provided a useful starting point for further larger-scale quantitative research to assess HPV awareness in Japanese men, as well as HPV vaccination uptake in girls of single fathers at the population level.

In conclusion, while knowledge about HPV among Japanese fathers is poor, HPV vaccination acceptability is high and does not differ by marital status. Providing adequate sexual health education in schools that addresses lack of knowledge about HPV as well as information preferences expressed by single fathers, may not only increase HPV vaccine acceptance, but will also actively involve men in cervical cancer prevention strategies. However, further large-scale quantitative studies are needed.

Acknowledgements

The authors wish to thank all the schools and parents who participated in the study. This study was funded by a Grant-In-Aid from the Japanese Ministry of Education, Culture, Sports, Science and Technology and in part by a Grant-in-Aid from the Japanese Ministry of Health, Labour and Welfare.

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PLOS ONE

Histone Deacetylase Inhibitors Sensitize Lung Cancer Cells to Hyperthermia: Involvement of Ku70/SirT-1 in Thermo-Protection

Mohamed K. Hassan[1,2]*, Hidemichi Watari[1]*, Alaa-eldin Salah-eldin[3¤a], Ahmed S. Sultan[4¤b], Zainab Mohamed[1], Yoichiro Fujioka[5], Yusuke Ohba[5], Noriaki Sakuragi[1]

1 Department of Obstetrics and Gynecology, Hokkaido University, Graduate School of Medicine, Sapporo, Japan, **2** Biotechnology program, Department of Zoology, Faculty of Science, Port Said University, Port Said, Egypt, **3** Department of Zoology, Aswan Faculty of Science, Aswan University, Aswan, Egypt, **4** Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, United States of America, **5** Department of Cell Physiology, Hokkaido University, Graduate School of Medicine, Sapporo, Japan

Abstract

This study describes the sensitization mechanism to thermal stress by histone deacetylase inhibitors (HDACIs) in lung cancer cells and shows that Ku70, based on its acetylation status, mediates the protection of lung cancer from hyperthermia (42.5°C, 1-6 hrs). Ku70 regulates apoptosis by sequestering pro-apoptotic Bax. However, its role in thermal stress is not fully understood. The findings showed that, pre-treating lung cancer cells with HDACIs, nicotinamide (NM) or Trichostatin A (TsA) or both significantly enhanced hyperthermia-induced Bax-dependent apoptosis in PC-10 cells. We found that hyperthermia induces SirT-1, Sirtuin, upregulation but not HDAC6 or SirT-3, therefore transfection with dominant negative SirT-1 (Y/H) also eliminated the protection and resulted in more cell death by hyperthermia, in H1299 cells through Bax activation. Hyperthermia alone primed lung cancer cells to apoptosis without prominent death. After hyperthermia Bax was upregulated, Bcl-2 was downregulated, the Bax/Bcl-2 ratio was inversed and Bax/Bcl-2 heterodimer was dissociated. Although hyperthermia did not affect total Ku70 expression level, it stimulated Ku70 deacetylation, which in turn could bind more Bax in the PC-10 cells. These findings suggest an escape mechanism from hyperthermia-induced Bax activation. To verify the role of Ku70 in this protection mechanism, Ku70 was silenced by siRNA. Ku70 silencing significantly sensitized the lung cancer cells to hyperthermia. The Ku70 KD cells underwent cytotoxic G1 arrest and caspase-dependant apoptosis when compared to scrambled transfectants which showed only G2/M cytostatic arrest in the cell lines investigated, suggesting an additional cell cycle-dependent, novel, role of Ku70 in protection from hyperthermia. Taken together, our data show a Ku70-dependent protection mechanism from hyperthermia. Targeting Ku70 and/or its acetylation during hyperthermia may represent a promising therapeutic approach for lung cancer.

Citation: Hassan MK, Watari H, Salah-eldin A-e, Sultan AS, Mohamed Z, et al. (2014) Histone Deacetylase Inhibitors Sensitize Lung Cancer Cells to Hyperthermia: Involvement of Ku70/SirT-1 in Thermo-Protection. PLoS ONE 9(4): e94213. doi:10.1371/journal.pone.0094213

Editor: Sue Cotterill St. Georges, University of London, United Kingdom

Received August 30, 2013; **Accepted** March 14, 2014; **Published** April 11, 2014

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Funding: This study was supported in part by a grant-in-aid HW for Scientific Research (C 22591844 and 20659255) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: mohamedkamel24@yahoo.com (MKH); watarih@med.hokudai.ac.jp (HW)

¤a Current address: Medical Laboratories Department, College of Science in AlZulfi, Majmaah University, Majmaah, Saudi Arabia
¤b Current address: Biochemistry Dept., Faculty of Science, Alexandria University, Alexandria, Egypt

Introduction

A long-standing research interest has been targeted the specific mechanisms responsible for the development of cancer cell resistance to different therapies. Targeting these mechanisms may enhance the specific destruction of cancer cells. Hyperthermia is a modality used in the clinical setting, for the treatment of many cancers; it is usually used in combination with radiotherapy and/or chemotherapy [1,2]. However, a significant obstacle to the effectiveness of hyperthermia is the development of cellular resistance, which blocks apoptotic signaling and enhances cell survival [3,4]. This resistance causes limitation of apoptosis after hyperthermia [5,6]. Thus, the identification of the mechanisms responsible for the development of thermo-resistance in cancer cells, might help improve specific targeting to enhance cellular sensitivity treatment outcomes to hyperthermia. Resistance to apoptosis is a common characteristic of cancer cells [3,7]. Apoptosis is induced by, extrinsic and intrinsic pathways [8]. Binding of ligands to a death receptor activates the extrinsic pathway; the intrinsic pathway is activated by cell stress, such as DNA damage. The Bcl-2 protein family regulates the intrinsic pathway; it influences the permeability of the outer mitochondrial membrane [9]. Members of the Bcl-2 family are divided into proapoptotic proteins such as Bax, Bak, and Bok, and anti-apoptotic proteins including Bcl-2, Bcl-xL, Bcl-w, and Mcl-1 [10–13].

Accumulation of Bcl-2 and Bcl-xL can protect cells from apoptosis, promote cell survival and accelerate tumor growth by sequestering pro-apoptotic Bax. Ku70 is another anti-apoptotic molecule; it naturally binds Bax, sequestering it from activation or

mitochondrial translocation in unstressed cells [14,15]. Ku70 is one of the components of the Ku70/Ku80 heterodimer that is involved in DNA damage repair [16]. Acetylation of two critical lysines, on the carboxyl terminus of Ku70, regulates the binding/dissociation to Bax and this affects the subsequent sensitivity of the cell to apoptotic stimuli [14]. Only deacetylated Ku70 can bind to Bax. High expression of Ku70 in cancer cells would enhance DNA repair ability and reduce Bax-mediated apoptosis; therefore, Ku70 might play a role in treatment resistance. The apoptosis-related activity of Ku70 is independent of its role in DNA repair [17]. The Ku70 acetylation/deacetylation cycle is regulated by histone acetyl transferases and histone deacetylases (HDACs). Ku70 is a target of some members of class I/II HDAC and class III HDAC [18,19]. The HDAC family of proteins is divided into two categories: zinc-dependent enzymes (HDAC1-11), subdivided into class I and class II which are inhibited by Trichostatin A (TSA) and NAD^+-dependent enzymes (class III; SIRT1-7) which is inhibited by nicotinimide (NAM). More precisely, SirT-1, a member of the class III HDACs, plays a crucial role in Ku70 deacetylation, which enhances the protection of cells from Bax during caloric restriction [19]. The majority of cancer cells over-express SirT1 [20]. Thus, targeting the Ku70-dependent protection from apoptosis, by HDAC inhibitors that inhibit SirT-1, could be an effective strategy for sensitizing cancer cells to different therapies. In this study, our model is that lung cancer cells are significantly killed by hyperthermia when pretreated with HDACIs compared with hyperthermia only. SirT-1 and its target, Ku70, are central to the mechanism by which lung cancer cells can escape thermal-induced death. Changes in the activity of Bax, Ku70 acetylation and the cell cycle were studied during exposure to hyperthermia. In addition, the efficiency of sequence specific targeting of Ku70, using siRNA, were also studied with regard to sensitizing lung cancer cells to hyperthermia. Ku70 appears to play a crucial role in the protection of cells from hyperthermia probably by sequestering up-regulated Bax.

Materials and Methods

Cell lines

Two human, non-small cell lung carcinoma cell lines: PC-10 [21], and H1299, were purchased (American Type Culture Collection, ATCC) and cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Sigma, St. Louis, MO, USA) (complete medium) in a humidified atmosphere of 5% CO_2 at 37°C. The cultured medium was replaced by fresh complete medium every three days.

Antibodies and reagents

Anti-human Bax rabbit polyclonal antibody (pAb) and anti-human Ku70 mouse monoclonal antibody (mAb) were purchased from BD Bioscience (Erembodegem, Belgium); another anti-human Ku70 mouse mAb for immunoprecipitation, anti-human HDAC-6 and anti-human SirT-3 rabbit (pAb) were purchased from Abcam (Cambridge, UK); anti-human Ku80 mouse mAb from Signal Transduction (USA), anti human SirT-1 and anti pan-K (Acetylated lysine) from Upstate (Upstate Biotechnology, Lake Placid, NY, USA); anti human Bcl-2 mouse mAb was purchased from DAKO (Glostrup, Denmark). Detection by immunoblotting was carried out with anti-mouse or anti-rabbit secondary HRP-conjugated Antibodies (Dako) diluted at 1: 2,000. Immunofluorescence staining was performed using anti-rabbit FITC-conjugated secondary Antibodies (Dako) diluted at 1: 50. Histone deacetylase inhibitors (HDACIs), Nicotinamide (NAM) and Trichostatin (TsA) were purchased from Wako Chemicals, Japan.

HDACI treatment optimization

Each HDACI used was screened for its sub-lethal dose in a pilot experiment. Cell viability was evaluated using the MTT assay, with or without DMSO only, and the addition of different doses of each of the HDACIs; 300 nM of TsA and 20 mM of NAM were chosen as non-toxic doses and further used in the subsequent experiments.

Heat treatment

The cells attached to the bottom of the culture dishes, were pre-culture for 48 h, and were then incubated in a humidified atmosphere of 5% CO_2 at either 37.0°C (control) or 42.5°C for 1–9 h.

Flow cytometry, cell cycle analysis and Annexin V staining

After exposure to hyperthermia, the cells were re-incubated at 37°C for 0 h, 24 h, or 48 h. Then, the cell cycle phases were analyzed. Briefly, the cells were fixed with 70% ethanol at 4°C overnight. After washing with Ca^{2+}-Mg^{2+}-free Dulbecco's PBS, the cells were treated with 0.1 mg/ml RNase (Type I-A; Sigma, St Louis, MO, USA) and then stained with 100 µg/ml propidium iodide (PI; Sigma), in the dark, at room temperature for 20 min. After passing through a 40 nm nylon mesh, the samples were kept on ice until measurements. The data obtained, using the FACS calibrator, were used to analyze the cell cycle phase proportions with ModFit software. A cell fraction of DNA, below the sub-G0/G1 peak, indicated apoptotic cells; DNA histograms were used for their estimation.

For Annexin V staining, the cells were directly stained with PI and Annexin-V Flous (Roche) for 10 min and then washed with incubation buffer. The cells were identified with a FACS calibrator after setting the voltage using non-treated stained control cells. The cells were analyzed using Cell Quest software and classified into four different stages: unstained living cells, early age apoptotic cells stained only with Annexin-V, middle age apoptotic cells doubly stained, and late age apoptotic and necrotic cells stained with PI only.

Immunoprecipitation

The cells (5×10^6/dish) were washed with cold PBS, lysed on ice in RIPA lysis buffer (50 mM Tris, pH 7, 150 mM NaCl, 0.5% sodium deoxycholate and .1% NP-40)(NP-40; Nacalai Teque, Kyoto, Japan) or CHAPS lysis buffer (150 mM NaCl, 10 mM HEPES, pH 7.4, 1% CHAPS) [22] supplemented with a protease inhibitor cocktail (Sigma) for 1 h, and then centrifuged at 15,000 rpm for 10 min. The supernatant was mixed with protein A-Sepharose (for pAb) or protein G-Sepharose (for mAb) (Amersham Pharmacia Biotech, Piscataway, NJ, USA), pre-swelled in PBS and pre-coated with the desired antibody against Bax, Ku70, Acetylated lysine or SirT-1 by gently shaking for 1 h at 4°C and centrifuged for 1 min at 3000 rpm. After washing with lysis buffer, the immunocomplex was fractionated by SDS-PAGE (10–12% gels) and then underwent Western blot analysis.

Western Blot Analysis

After SDS-PAGE, the proteins were transferred to a PVDF membrane (Amersham, Buckinghamshire, UK). The membrane was blocked at 4°C overnight with blocking buffer. The membrane was incubated for 1 h at room temperature with the desired Ab. After washing three times with TPBS, the membranes were incubated for 1 h with secondary Ab, at room temperature, followed by three washes with TPBS. The membrane was

developed using ECL reagents (Amersham). The chemiluminescence was visualized with a polaroid camera (Amersham Pharmacia) and quantified using densitometry.

siRNA design and transfection

siRNA oligomers against Ku70 mRNA (Ku70-siRNA) and a control sequence that did not match any gene sequence (Cont-siRNA) were either purchased as a validated one (Ambion, USA; Ku70-siRNA-2 and cont-siRNA-2, respectively) or designed by the investigators and then synthesized by Ambion according to the following sequence: Ku-siRNA, 5_UUCUCUUGGUAA-CUUUCCCdTdT_3 (Ku70-siRNA-1) and 3_dTdTAAGA-GAACCAUUGAAAGGG_5; Cont-siRNA, 5_GCG CGC UUU GUA GGA UUC GdTdT_3 and 3_dTdTCGCGCG AAA CAU CCU AAG C_5 (cont-siRNA-1). This sequence targeting was validated [23]. siRNA oligomers against Bax mRNA (Bax-si) was purchased from Qiagene (Cat No. SI04948202). The Bax-si, the Ku70-siRNAs or cont-siRNAs were transfected into the lung cancer cells (10^5 cells/60-mm dish) using SiPORT *Neofex* (Ambion; USA) to a final concentration of 200 nM, two times. One day after the last transfection, the cells were trypsinized and plated onto 60 mm dishes (50.000 cells per dish) in triplicate. After cell attachment, the cells were exposed to hyperthermia at the indicated time intervals according to the experimental design. Each experiment was repeated at least three independent times for reproducibility and statistical calculation.

Statistical evaluation

Statistical analyses were performed using Minitab Release (Ver.12). Data are expressed as the mean ± S.E.M. One way analysis of variance (ANOVA) was used to assess the statistical significance between means. Differences between means were considered significant at p-values less than 0.05.

Results

HDACIs significantly facilitated cell death with hyperthermia

As a fact, SirT-1, a human deacetylase, was specifically targeted by small molecules known as HDACIs, for example NAM. Moreover, Ku70 deacetylation was inhibited by inhibitor molecules that target the class I/II HDAC; for example TsA. We first pre-treated the PC-10 cells with different HDACIs, NAM (20 mM), TsA (300 nM) or both, for 4 h right before exposure to hyperthermia. This combined treatment significantly increased apoptosis, more than two folds, compared with hyperthermia treatment alone, as observed by phase contrast microscopy (Figure. 1A) and Annexin V staining (Figure. 1B). The Bax expression pattern was analyzed in whole cell lysates from treated and untreated PC-10 cells. Bax expression (21 kDa) was increased by hyperthermia. Interestingly, HDACIs (NAM and TSA) increased the production of 18 kDa Bax, which is known to be a N-terminus truncated form of Bax. Given that Bax activation is induced by either the N-terminus exposure by conformational change (reversible change) or N-terminal truncation (irreversible change), these findings suggest that sustainable enhanced apoptosis with hyperthermia and HDACIs is Bax-dependent and this apoptosis may depend on upregulation of Bax or the release of Bax from antiapoptotic protein(s) to promote apoptosis (Figure. 1C). Importantly, the treatment of lung cancer cells with HDACIs, only at selected doses, had no appreciable toxic effect. Similar effects of HDACIs were observed in the H1299 cells (Figure. S1 and S2). Unexpectedly, the triple combination of NAM, TsA and hyperthermia was less effective in H1299 than dual combination. Although the exact molecular mechanism of this phenomenon remains obscure, one possible reason is that the triple combination may enhance the division of the surviving cells escaped the challenge of triple treatment, which can yield the production of new daughter cells within the 48 hours post treatment (before annexin V staining detection) and cause the reduction of the annexin V staining percentage finally. To verify that Bax plays the major role in hyperthermia-induced apoptosis after targeting Ku70 deacetylation, Bax was targeted by specific siRNA in PC-10 cells (see materials and methods). Bax was amenable to siRNA transfection (Bax-si) when compared with control scrambled oligo siRNA (cont-si) as detected by western blot analysis (Figure. 1D; upper panel). Again, when Bax-knocked down PC-10 cells were treated with hyperthermia for 6 h in the presence of HDACIs, the hyperthermia-induced cell death was significantly inhibited (Figure. 1D; lower panel). Meanwhile, cell death was not completely blocked under hyperthermia treatment alone (Figure. 1D; lower panel and Figure. S3). This result may indicate the involvement of other pro-apoptotic molecules in the limited cell death induced by hyperthermia.

Effect of hyperthermia on expression of apoptosis related proteins in PC-10 cells

To investigate the effect of hyperthermia on Bax and its major binding molecules, some of the apoptosis-related proteins (Bax, Bcl-2, Ku70 and Ku80) were studied by western blot analysis in a representative cell line, PC-10. Because most of the housekeeping genes are responsive to hyperthermia, thus Ponceau S staining was used to show the loading control. Hyperthermia (42.5°C) for 1–9 h induced quantitative changes in Bax and Bcl-2 expression, with no observable changes in Bcl-xL, Ku70 and Ku80 (Figure. 2A) Bax expression was slightly up-regulated while Bcl-2 expression was down-regulated in PC-10 cells. The Bax/Bcl-2 ratio gradually increased after hyperthermia treatment (Figure. 2B, upper panel). Western blot analysis of Bax and Bcl-2, with different loading amounts of protein (20 μg, 40 μg and 60 μg/lane), after 0 h and 6 h hyperthermia treatment confirmed an increased Bax/Bc-l2 ratio (Figure. 2B, lower panel) as verified denstimetrically (using Scion Image software). Similar observations were also obtained in H1299 cells (Figure. S4). Hence, we had much interest to answer the question why cancer cells, with wild-type Bax, which was upregulated, did not show prominent apoptosis after hyperthermia unless DHACIs are added. Noticeably, by immunocytochemistry Bax and Bcl-2 showed mainly cytosolic localization in the cell lines studied (Figure. S5). Therefore, we moved to study Bax dimerization.

Disturbance of Bax heterodimerization by hyperthermia

In unstressed cells, Bax heterodimerizes with many, anti-apoptotic, partner molecules; it homodimerizes under stress to induce apoptosis. To study the effect of hyperthermia on Bax dimerization, Bax was immunoprecipitated from the PC-10 cell lysate after 6 h exposure to hyperthermia. Figure.3A, shows decreased Bax/Bcl-2 heterodimer formation; the Bax/Bcl-xL heterodimer was not affected by hyperthermia, according to the results of the Western blot analysis. Of note, was that the amount of Ku70 bound to Bax increased with increased exposure time to hyperthermia. The Ku70 immunoprecipitation confirmed enhanced Bax/Ku70 binding after 6 h exposure to hyperthermia, while Ku70/Ku80 binding showed no change (Figure. 3B). Similar observation was detected when CHAPS buffer was used for the immunoprecipitation experiments (Figure. S6)

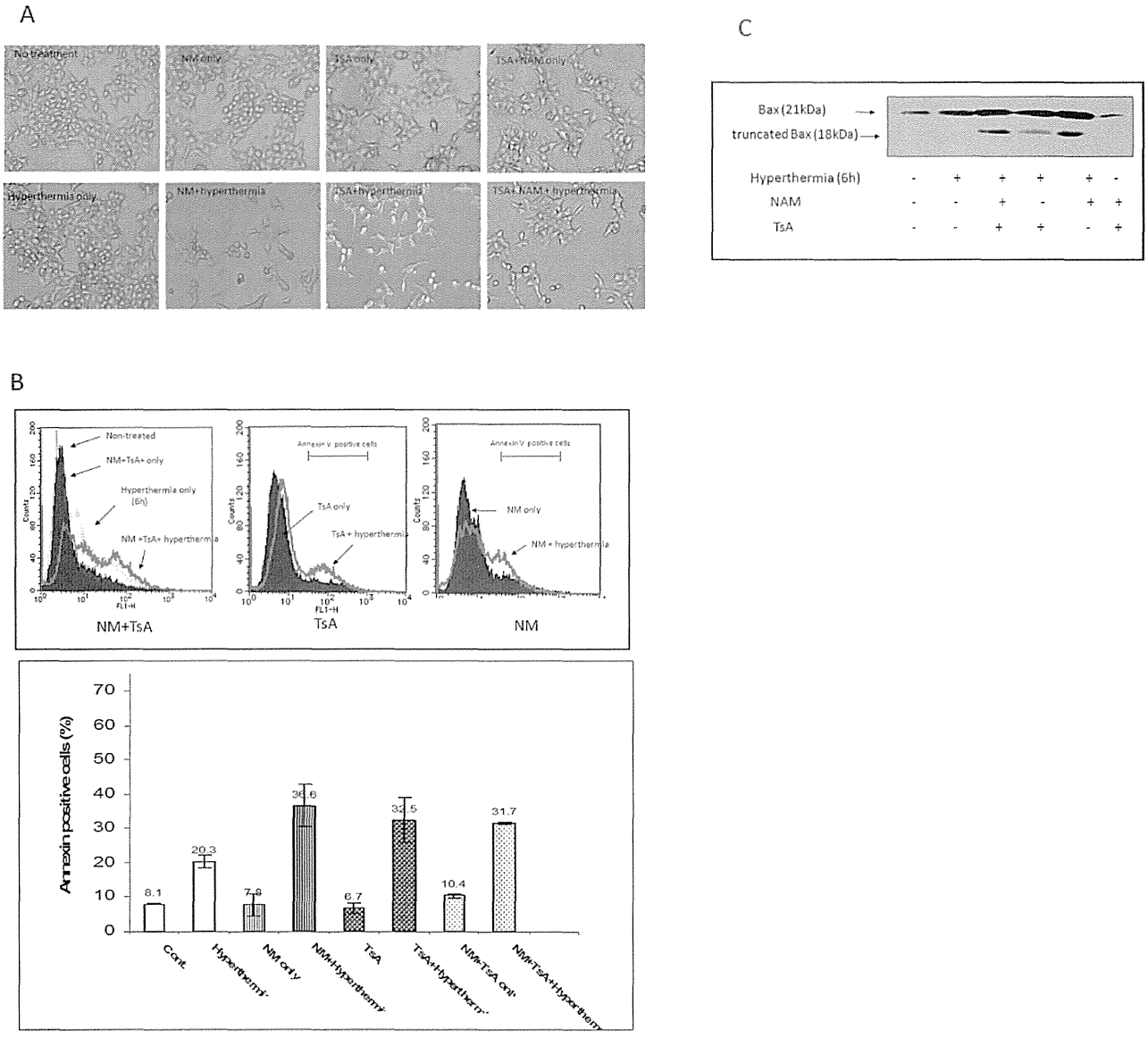

Figure 1. *A.* Phase contrast photographs of PC-10 cells, two days after treatment with HDACIs then- hyperthermia for 6 h. The image showed limited number of recovered cells as well as the rounded and floating apoptotic cells in the colonies pre-treated with HDACIs then hyperthermia. *B.* Pre-treatment of PC-10 cells with HDACIs significantly increased hyperthermia-induced apoptosis. Comparative cytograms show Annexin V staining after hyperthermia in PC-10 cells pre-treated with nictotinamide (20 mM), Trichostatin A (300 nM) or both (upper panel). Summary of average annexin V results is concluded (lower panel). *C.* Bax expression levels, by Western analysis, in PC-10 cells after hyperthermia (6 h) pre-treated with different HDACIs. Lower band (18 KDa) indicates truncated, active, Bax only increased when cells treated with combination of HDACIs and hyperthermia. *D.* A representative western blot shows the amendment of Bax for the specific siRNA used. PC-10 cells were either transfected with Bax-si or cont-si twice. Actin immunoblot was used as a loading control (upper panel). Significant reduction of annexin V staining after hyperthermia and HDACIs in Bax KD cells compared with control(s) indicating that Bax is the key proapoptotic player in the double treatment-induced apoptosis (lower panel). Each data point represents the mean of three experiments; bars denote SD; ** indicates difference from control transfectant at $P<0.01$.
doi:10.1371/journal.pone.0094213.g001

Hyperthermia reduced Ku70 acetylation in the PC-10 cells

Acetylation of either K539 or K542 at the Ku70 C-terminal linker is sufficient to completely block Ku70 suppression of Bax-mediated apoptosis [14]. The effects of hyperthermia on the Ku70 acetylation status were therefore investigated by probing the blot of Ku70 immunoprecipitant with antiacetylated lysine Ab (Figure. 4A); the results show a significant reduction in Ku70 acetylation after 6 h exposure to hyperthermia in PC-10 cells. In addition, the amount of Ku70 detected, in the acetylated protein immunoprecipitant, decreased in a time-dependent manner (Figure. 4B).

SirT-1 mediates Ku70-dependent cytoprotection from hyperthermia

It is well known that Ku70 acetylation is specifically reversed by SirT-1, a human deacetylase, under mild stress (such as, caloric restriction); Under such conditions, Ku70 sequesters more Bax

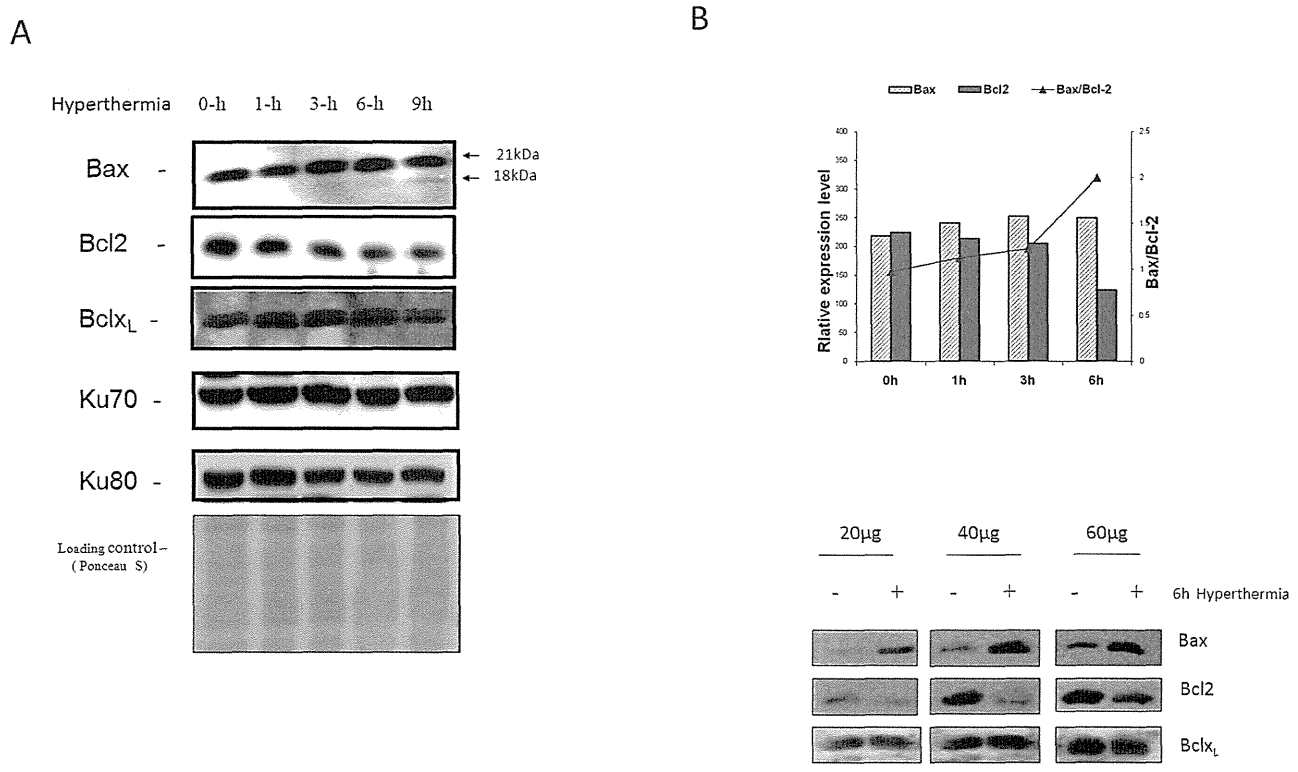

Figure 2. Hyperthermia affects expression levels of different apoptosis-related proteins. *A.*Whole cell extracts were prepared at indicated periods after 42.5°C hyperthermia and Bax, Ku70, Ku80 and Bcl-2 were analyzed by immunoblotting. Slight increase in Bax only after 6 and 9 h hyperthermia and limited Bax activation after 9 h are observable. Hyperthermia reduced Bcl-2 while Bcl-xL and Ku70 had not been clearly affected. Anaysis was performed in the same blot so each protein worked as a loading control for the other. A representative Ponceau S staining of the membrane was shown to verify the normalization because most of the basic house-keeping genes (e.g: actin and tubulin) are responding to hyperthermia. *B.* Quantitative analyses of Bcl-2 and Bax protein expression during hyperthermia. Each band was quantified densitimetrically. A representative set of Bax/Bcl-2 ratio is shown. Western analysis with different loading amount of PC-10 cells lysates after 6 h hyperthermia verifies the change in the Bax/Bcl-2 ratio, while Bcl-xL expression was used as a loading control from same blot.
doi:10.1371/journal.pone.0094213.g002

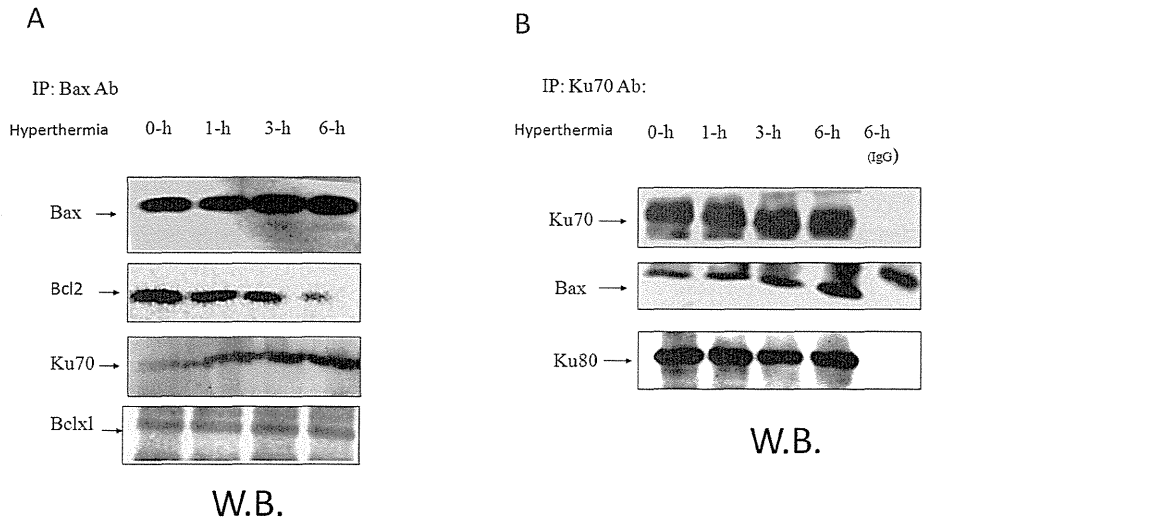

Figure 3. Hyperthermia modulates Bax association with Ku70 and Bcl-2. *A.* PC-10 cells were incubated at 42.5°C for 0, 1, 3 and 6 h. Bax was co-immunoprecipitated from 2 mg total protein and Bcl-xL, Bcl-2 and Ku70 were detected in the immunoprecipitant by western analysis. Hyperthermia induces Bax up-regulation and Bax dissociation from Bcl2 and enhances association between Bax and Ku70, while no effect on the Bax/Bcl-xL association. In contrast, Ku70 was co-immunoprecipitated from similar cell lysates. Bax and Ku80 are shown in the immunoprecipitant. *B.* After hyperthermia, total Ku70 levels showed no changes, but association between Ku70 and Bax was enhanced.
doi:10.1371/journal.pone.0094213.g003

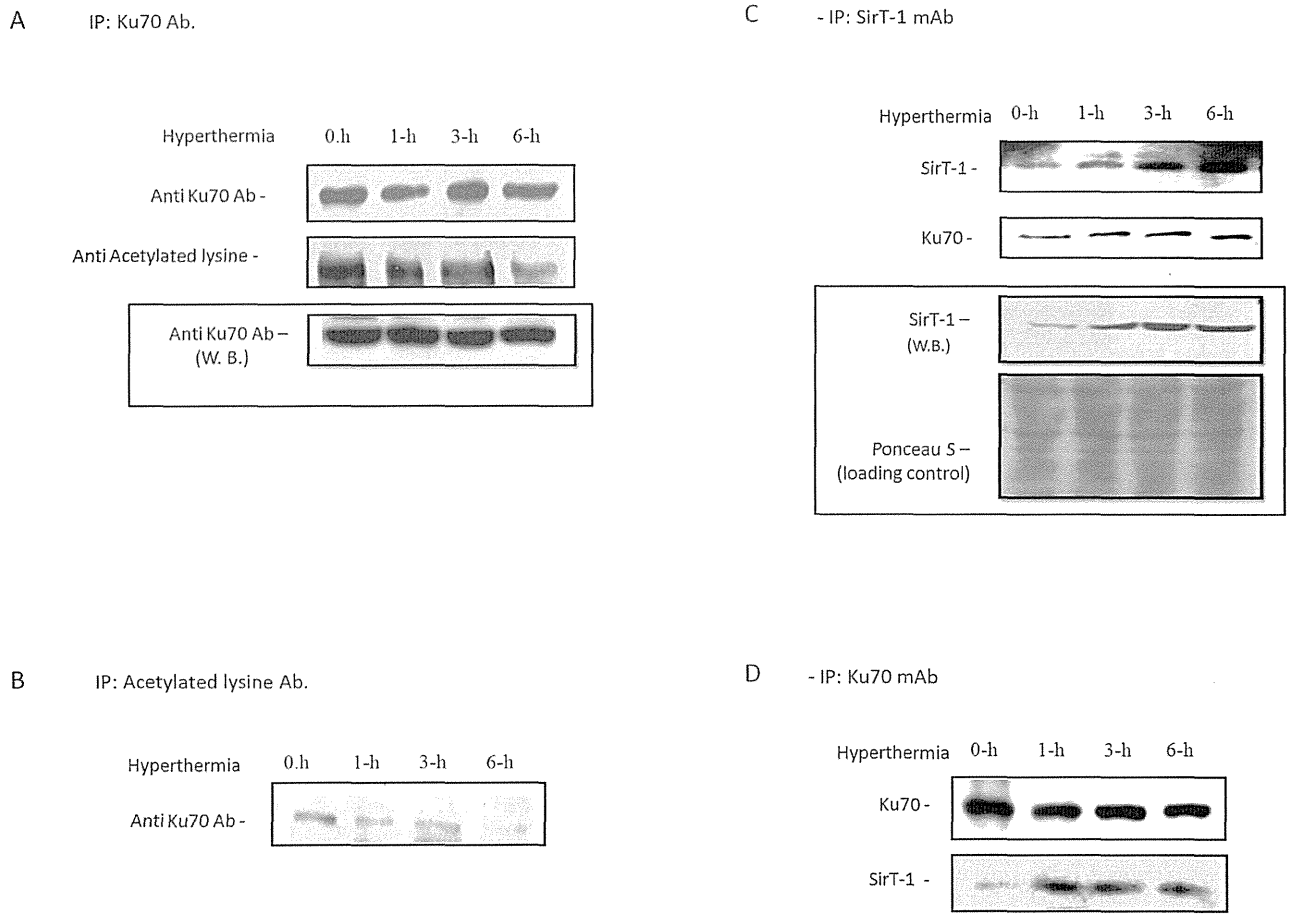

Figure 4. Hyperthermia treatment reduced Ku70 acetylation. *A.* Ku70 was immunoprecipitated from equal protein amounts (3 mg) of PC-10 cell lysat after hyperthermia at indicated time. Ku70 and acetyl Lysine were detected in the immunoprecipitant. Total Ku70 showed no change after 6 h hyperthermia (in put) but acetylated Ku70 was decreased. *B.* All acetylated proteins were immunoprecipitated, fractionated, blotted and Ku70 was detected in the blot. Bands indicated acetylated Ku70 became fainter with increasing hyperthermia time. *C.* Hyperthermia enhanced both Ku70 expression and Ku70/SirT-1 binding. SirT-1 was immunoprecipitated from PC-10 after hyperthermia (0–6 h). Blot indicates SirT-1 up-regulation (in put; lower panel) and enhanced binding to Ku70, in the same blot, was up-regulated (upper panels). *D.* Similar hyperthermia treatment in H1299. Total Ku70 was precipitated. Ku70 and SirT-1 were detected in the immunoprecipitant by Western analysis. SirT-1/Ku70 binding was increased, indicating an enhanced Ku70 deacetylation in lung cancer cells by hyperthermia.
doi:10.1371/journal.pone.0094213.g004

and protects cells from Bax-mediated apoptosis [19]. To investigate whether acetylation inhibition, with exposure to hyperthermia, was due to activation of SirT-1, we analyzed the SirT-1 expression after exposure to hyperthermia (0–6 h). Western blot analysis revealed that SirT-1 expression was induced by hyperthermia in PC-10 cells (Figure. 4C). Further, SirT-1 was immunoprecipitated from PC-10 whole cell lysates and then subjected to Western blotting analysis. The amount of Ku70 immunoprecipitated with SirT-1 was enhanced by exposure to hyperthermia (Figure. 4C). Similarly, the amount of SirT-1 immunoprecipitated with Ku70 was also enhanced by exposure to hyperthermia (0–6 h) confirming that Ku70/SirT-1 binding was enhanced by hyperthermia in PC-10 cells (Figure.4D). We speculated that up-regulated SirT-1, under conditions of hyperthermia, binds to Ku70 and changes it from acetylated to deacetylated form, which allows Ku70 to sequester more Bax, either liberated from Bcl2 or newly induced under hyperthermia, to inhibit hyperthermia-induced apoptosis finally. These results explain, at least in part, why HDACIs, such as NAM, can enhance apoptosis by hyperthermia, probably by targeting some HDACs like SirT-1. Notably, other histone deacetylases including HDAC6 and SirT-3, did not show significant changes in the expression profile after exposure to hyperthermia (Figure. S7). To confirm the above results, H1299 cells (with relatively high transfection efficiency, >50% as determined by Beta gal transfection) were transiently transfected either with wild-type SirT-1 or dominant negative H363Y/SirT-1. Hyperthermia-induced apoptosis was significantly enhanced in the H363Y/SirT-1-transfected cells compared to control vector transfectants (Figure. 5A; $P<0.01$). Importantly, the wild-type SirT-1-transfected cells showed slight but significant ($P<0.05$) protection from hyperthermia in the tested cells, indicating that the anti-apoptotic effect of the exogenous SirT-1 is significant but limited. This was likely because the endogenous SirT-1 had triggered most of the spontaneous protection from hyerthermia as indicated by the DNA content experiments (Figure. 5A). Similar results were obtained from the Annexin V staining; H363Y/SirT-1 transfection into H1299 cells significantly enhanced apoptosis after exposure to hyperthermia compared to the empty vector transfectant (Figure. 5B). Notably, neither wild-type SirT-1 nor

dominant negative H363Y/SirT-1 transfection individually showed appreciable changes in the cell cycle.

Targeting Ku70 by siRNA enhanced hyperthermia-induced cell death

To examine whether Ku70 was a key mediator in the aforementioned protection of the cells from hyperthermia, Ku70 mRNA was targeted by sequence specific Ku70-siRNA-1,-2. Ku70 mRNA was amenable to Ku7-siRNA-1 (custom design) and subsequently, the level of protein expression was significantly reduced (dose; 200 nM) in PC-10 cells (Figure. 6A). The control siRNA (cont-siRNA-1) transfection did not affect Ku70 expression. In addition, Ku70-siRNA-2 transfection (see materials and methods) was confirmed to efficiently knockdown Ku70 (Figure. 6A, lower panel). Next, both the Ku70 knockdown (KD) and control cells were challenged with exposure to hyperthermia. The Ku70 KD PC-10 cells showed enhanced cell death after hyperthermia exposure in a time-dependent manner (Figure. 6B, C) compared to the cont-siRNA transfectant, two days after treatment, as indicated by the FACS analysis (using Ku70-siRNA-1). Similar results were obtained with the use of H1299 cells (using Ku70-siRNA-2 and cont-siRNA-2 (Figure. S8)). These results suggest that Ku70 mediates cytoprotection from hyperthermia exposure and likely plays a key role in hyperthermia-induced apoptosis.

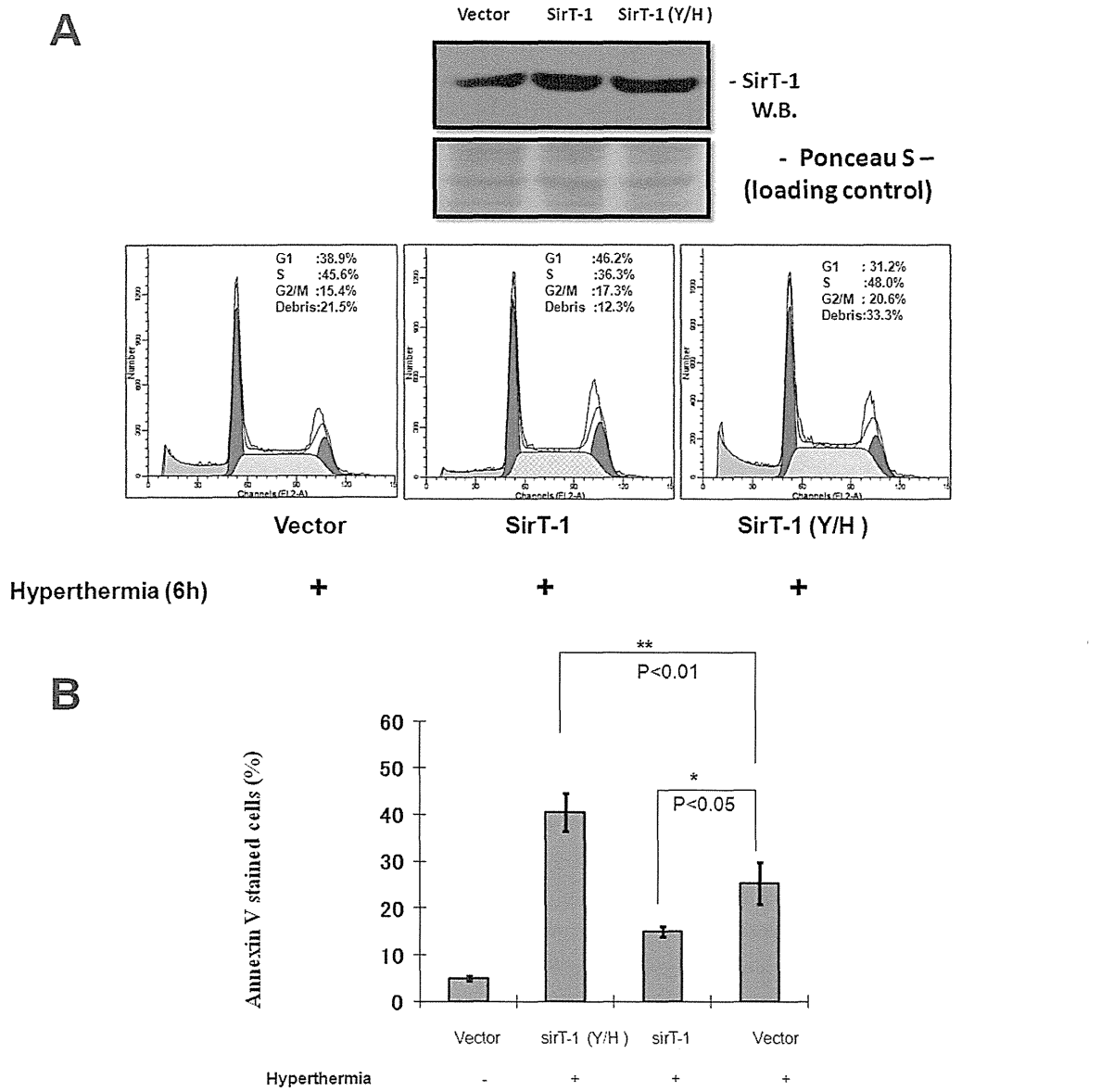

Figure 5. Dominant negative SirT-1 (H363Y) and HDACIs enhanced hyperthermia-induced cell death. *A.* SirT-1 is involved in the cytoprotection from hyperthermia. Western blot shows the over-expressed SirT-1. FACS analysis of H1299 cells after transient transfection either with wide type SirT-1, dominant negative SirT-1 or FLAG expression vector. H363Y/SirT-1 significantly enhanced hyperthermia-induced cell death while wide type SirT-1 had no significant effect (upper panel). *B.* Results of annexin V staining of H1299 cells, from similar experiment, confirming that H363Y/SirT-1-induced cell death is apoptosis (lower panel).
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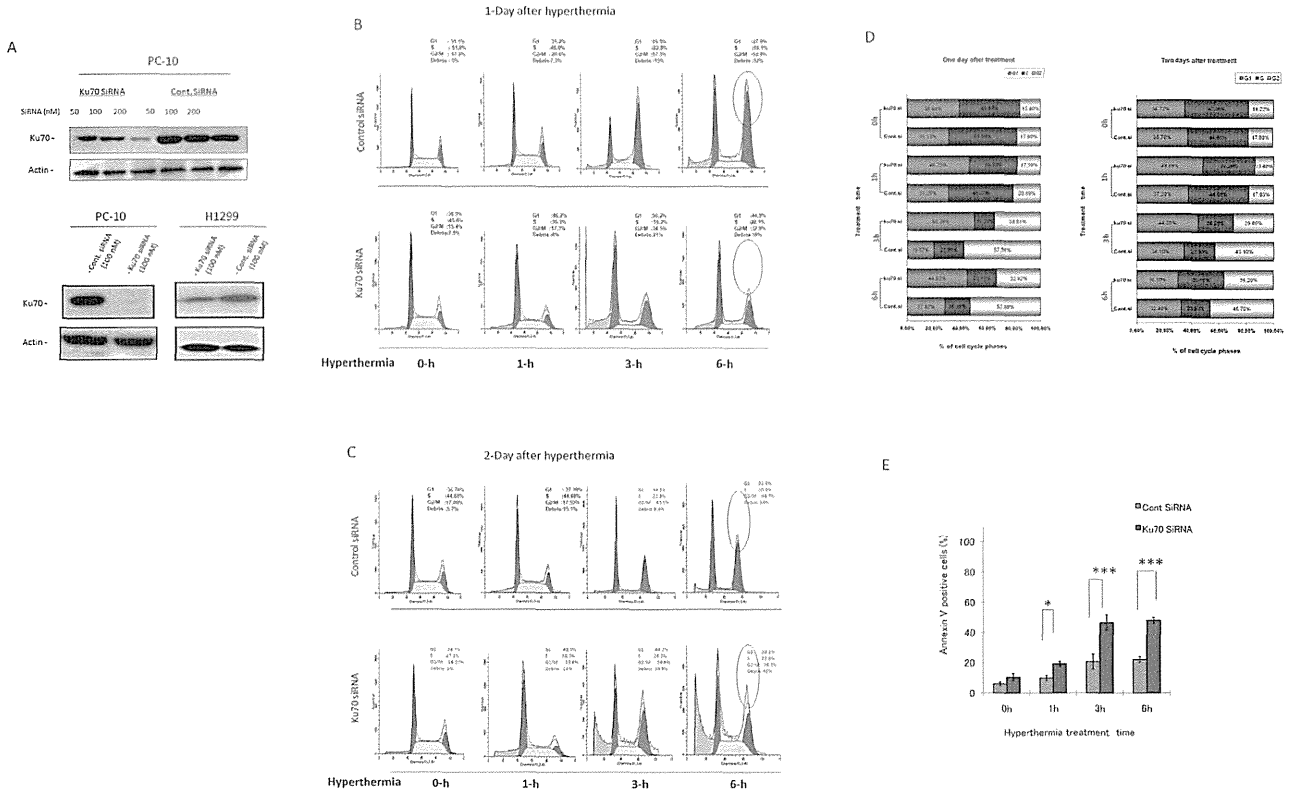

Figure 6. Ku70 knock down enhanced apoptotic cell death after hyperthermia in PC-10. *A.* A representative western blot shows the amendment of Ku70 for both siRNAs used. PC-10 cells and H1299 cells were transfected by one siRNA twice. The western result shows significant knock down by Ku70-siRNA-1,-2 comparing with the cont-siRNA-1,-2, respectively. Actin immunoblot was used as a loading control. The experiment was repeated three independent times for reproducibility. PC-10 cells were transfected with Ku70-siRNA-1 or cont-siRNA-1 (200-nM) twice. 24 h after last transfection, equal cell numbers were subcultured for further 24 h, and then treated with hyperthermia for indicated time intervals, and then re-cultured at 37°C for 24 h (B) or 48 h (C) Cells were acquired by FACS analyzer for cell cycle analysis. Results shown are a representative one from, at least, three independent experiments for each time point (24 h and 48 h). *D.* Ku70 is required for cytostatic arrest by hyperthermia. Histograms show the differential accumulation of cell populations into G2/M phases in cells with or without Ku70, one and two days after 0, 1, 3 and 6 h hyperthermia treatment. Populations in different cell cycle phases, G1, S and G2/M phases, were calculated using computer after hyperthermia treatment. Results shown are average from three independent experiments. *E.* Significant enhancement of annexin V staining after hyperthermia in Ku70 KD cells compared with control indicating that Ku70 silencing-based cell death by hyperthermia is apoptosis. Each data point represents the mean of three experiments; bars denote SD; * indicates difference from control transfectant at $P<0.001$.
doi:10.1371/journal.pone.0094213.g006

Ku70 mediated hyperthermia-induced G2/M accumulation

Pulse-labeling experiments with bromodeoxyuridine (BrdUrd; 20 μM) in PC-10 cells indicated that exposure to hyperthermia induced cytostatic but not cytotoxic arrest (data not shown). The hyperthermia-induced cell cycle disturbance was analyzed 24 h and 48 h after 1–6 h exposures to hyperthermia. G2/M subpopulations were significantly increased 24 h after exposure to hyperthermia and then gradually decreased to normal subpopulations 48 h after exposure to hyperthermia (Figure. 6D). Simultaneously, the percent of G1 and S phase subpopulations gradually decreased 24 h after exposure to hyperthermia and recovered 48 h after the removal hyperthermia. Different from the control cells, the Ku70 KD PC-10 cells did not show G2/M accumulation 24 h after exposure to hyperthermia, but directly underwent G1 cytotoxic arrest that resulted in apoptosis without significant recovery (Figure. 6B, C, and D). These data suggest that Ku70 was required for cytoprotective cytostatic arrest during the G2/M phase after exposure to hyperthermia.

Hyperthermia-induced cell death in Ku70 KD cells was a caspase-dependent apoptosis

Annexin V staining confirmed that Ku70 silencing-induced cell death, under conditions of hyperthermia, is apoptosis (Figure. 6E). Similar results were obtained with siRNA transfection into another lung cancer cell line, H1299 (data not shown). To investigate whether the enhanced apoptosis in the Ku70 KD cells was caspase-dependent, both Ku70 KD and control PC-10 cells were exposed to hyperthermia (6 h) in the presence or absence of a caspase inhibitor, v-DEVD-Fmk (z-VAD) and then assessed by FACS analysis to determine their apoptotic status. Figure. 7A shows a significant reduction of hyperthermia-induced apoptosis, especially in the Ku70 KD cells. Western blot analysis of some apoptosis-related proteins in the Ku70 KD and control cells, showed significant reduction of Ku70 levels in the Ku70 KD cells compared to the control cells (Figure. 7B). The Bax levels were up-regulated in both cells in response to hyperthermia whereas Bax activation (highly migrating truncated band; 18 kDa) was only observed in the Ku70 KD cells. The active level of Bax was increased with the hyperthermia treatment time. P53 expression was down-regulated after exposure to hyperthermia in both clones

(Figure. 7B; far lower panels); these results suggest that Bax up-regulation was independent of P53 as confirmed in H1299 cells which express no functional P53. Figure. 7C concludes the possible protection mechanism by Ku70 and interprets how HDACIs disturb this mechanism.

Discussion

The results of this study suggest a candidate mechanism responsible for resistance to hyperthermia-induced apoptosis in lung cancer cells. As a fact, Bcl-2 heterodimerizes with Bax to inhibit its apoptotic effects [24]. Thus, the Bax/Bcl-2 ratio reflects apoptosis susceptibility [25]. However, Bcl-2 and Bax function independently to regulate cell death [26]. Although hyperthermia can activate some caspases [27], when hyperthermia is used clinically for cancer treatment, hyperthermia-induced apoptosis has very limited effects. This is consistent with our observation that Bcl-2 was down-regulated while Bax was up-regulated, without prominent Bax activation, in the cells studied. The Bax/Bcl-2 ratio increased under conditions of hyperthermia.

It is well known that Ku70 plays a dual role in DNA double strand break (DSBs) repair and in suppressing Bax-mediated apoptosis, by interacting with Ku80 and Bax [28,29]. However, in the absence of DNA breaks, it is not known whether Ku70 inhibits apoptosis by associating only with Bax or by mediating other pathway(s) that affect Bax. The recently reported cytoprotective function of Ku70 is based on deacetylation [14,17,29], that renders cancer cells more susceptible to DNA damaging agents or to Bax activating factors and that are affected by targeting acetylation. The results of this study showed that the total amount of Ku70 did not significantly change; however, Bax/Ku70 binding was increased with exposure to hyperthermia. The Ku70 binding to Bax might be due to either increased Bax expression or Bax liberated from Bcl-2. The acetylation status of Ku70 changed with exposure to hyperthermia.

Ku70 acetylation, by both the I/II HDACs and class III/Sirtuin deacetylases, including SirT-1, has been previously reported [17,30]. The results of this study demonstrated that, SirT-1 was directly up-regulated and interacted with Ku70, under conditions of hyperthermia, resulting in deacetylation, and the subsequent ability to sequester more Bax. This scenario might be one plausible mechanism associated with the promotion of cell survival. SirT-1, was reported to deacetylates specific lysine residues of many substrate proteins including Ku70 [31]. SirT-1 was consistently found to mediate survival with exposure to stress [32]. Ku70 is acetylated by p300, PCAF, and CBP. This acetylation process accelerates Bax-mediated apoptosis [14]. Ku70 deacetylation has been shown to contribute to longevity under conditions of caloric restriction [19]. Ku70 acts to sequester Bax from mitochondria [14,15]. Here, we found that SirT-1 and Ku70 work together to modulate thermo-sensitivity by regulating Ku70 acetylation. This is consistent with the reports describing SirT-1 as a responder to environmental stress [20,32]. The results of this study demonstrated that the inhibition of Ku70 deacetylation, by specific HDACIs, NAM or TsA, attenuated the protective role of SirT-1 from hyperthermia and enhanced hyperthermia-induced apoptosis.

Lung cancer cells were sensitive to Ku70 siRNA-based inhibition. This inhibition interfered with the protective mechanism against hyperthermia, and resulted in significant apoptosis. The DNA content, annexin-V staining and morphological changes observed, all confirmed induced apoptosis. Bak and/or Bax activation is necessary for intrinsic apoptosis [33]. Bax activation is essential and sufficient for mitochondrial permeabilization and cytochrome C release [33,34]. The results of this study demonstrated Bax activation after exposure to hyperthermia in Ku70 KD cells; in addition, apoptosis was blocked by treatment with the apoptosis inhibitor, z-VAD. The findings of this study showed that heat stress-induced apoptosis takes several hours in cells compared to minutes in isolated mitochondrial systems [35,36]. The antiapoptotic proteins, Bcl-2 and Bcl-xL, can sequester activator proteins and inhibit their ability to homodimerize and regulate apoptosis. Ku70 can be added to the list of antiapoptotic molecules that sequester Bax and inhibit its activation after exposure to hyperthermia ex vivo. Hyperthermia primed the signal for apoptosis by increasing the expression of Bax. However, the direct activation of Bax by hyperthermia may either require more exposure time or special conditions. Among these conditions are the addition of HDACIs to culture media to induce Bax/Ku70 flipping and Bax-based apoptosis. The combination (hyperthermia and HDACIs) treatment-induced apoptosis was significantly inhibited in Bax KD cells, which indicates the crucial role of Bax in this cell death. Rather than "death by default," the emerging view is that apoptosis requires Bax activation, which can be achieved by targeting Ku70 deacetylation with HDACIs or Ku70 knockdown during exposure to hyperthermia.

Human lung cancer cells are thermo-sensitive [36]. Hyperthermia may induce double strand DNA breaks [37]; however, only limited cell death occurs with hyperthermia independent of DNA breaks [38]. Although hyperthermia destroys some cells, by an unknown mechanism, hyperthermia selectively induces apoptosis during the S-phase in lung cancer cells [39]. This is probably because the S-phase and M-phase are the most sensitive to the cell death program [40].

Many studies, including this one, have shown that hyperthermia results in temporary (cytostatic) arrest of most cancer cells in the G2/M phase [41]. A majority of such cells re-enter the cell cycle after removal of the thermal stress and limited apoptosis occurs. The results of this study showed that Ku70 silencing inhibited the cytostatic effects of hyperthermia and caused cytotoxic G1 accumulation. These findings suggest that Ku70 is essential for G2/M accumulation and subsequent protection from hyperthermia. Yamamoto et al., [42], reported that cells are more susceptible to a death signal during G2/M because of Bcl-2 phosphorylation, which lowers the apoptosis threshold. The results of this study did not show Bcl-2 phosphorylation but rather only down-regulation under conditions of hyperthermia; these findings indicate that Bcl-2 does not play a role in protection from hyperthermia ex vivo. Instead, Ku70 was mainly involved in the protection scenario. Changing the acetylation status of cells may influence chromatin condensation and hence, DNA-repair. Deacetylation of a critical component of DNA repair machinery such as Ku70 or Ku70 KD may affect DNA repair machinery; however, again, cell death by hyperthermia had not been attributed to DNA break [39]. Even though the Ku70 KD cells, in this study, did not show abnormal cell cycle patterns without stress. These findings indicate that Ku70 plays a key role in the cell cycle progression that is essential for protection from hyperthermia. Thus, targeting Ku70, rather than inhibiting its deacetylation, may facilitate cell toxicity under conditions of hyperthermia, by a mechanism that is associated with a cell cycle-dependent disturbance.

Conclusion

In summary, the main finding of this study was the biphasic role of Ku70 during thermal stress. This finding might have

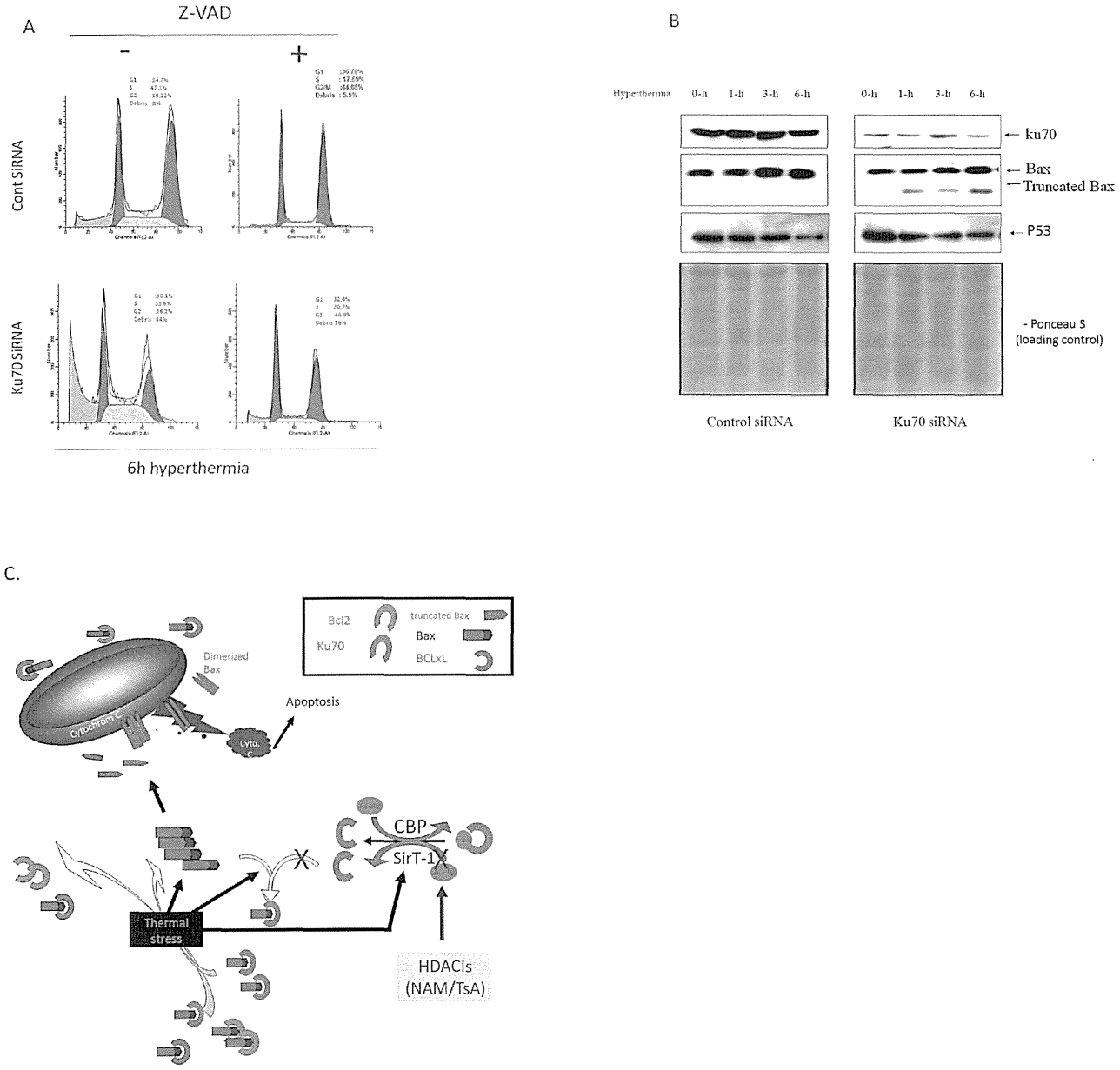

Figure 7. Enhanced cell death in Ku70 KD cells is caspase-dependent. *A.* Ku70 KD and control PC-10 cells were treated with hyperthermia (42.5°C) for 6 h in presence or absence of caspase inhibitor (Z-VAD; 50 nM). Two days later, cells were fixed and acquired by FACS analyzer. Cell death by hyperthermia in both cell clones was regressed by caspase inhibitor indicating that hyperthermia kills Ku70 KD and control cancer cells through caspase-dependent apoptosis. *B.* Proteomic profile of some apoptosis-related proteins in Ku70 KD cells. Ku70 KD and control PC-10 cells treated with hyperthermia at indicated time, lysed, fractionated and blotted. Ku70, Bax and P53 were detected in the blots. Right panel, Ku70 clearly knocked down. Bax shows extra band around 18 kDa indicating active Bax only in the Ku70 KD cell but not the control transfectant, left panel. The active band increased with increasing hyperthermia treatment time. P53 decreased by hyperthermia in both clones indicating that hyperthermia-induced Bax up-regulation and activation are P53 independent. **C.** Schematic presentation describes role of Ku70 in cellular protection from hyperthermia. Ku70, like Bcl-2 and Bcl-xL, restrains Bax from translocation into mitochondria in cells without stress. Under hyperthermia application, Bcl-2 is down-regulated and some Bax become free. In addition, more Bax is overexpressed by hyperthermia. Despite Bax regulation, no Bax is activated because Ku70 bind with this free Bax. Total Ku70 has change but acetylated Ku70 transformed into deacetylated due to SirT-1 activation. Addition of HDACIs during hyperthermia sensitized lung cancer cells to hyperthermia. HDACIs inhibited SirT-1 function and subsequently increase the chance of Bax activation and translocation to mitochondria inducing apoptosis under hyperthermia.
doi:10.1371/journal.pone.0094213.g007

therapeutic relevance, with regard to the interplay between Ku70 acetylation and/or expression as a modulator of subsequent Bax activation. The results of this study add to the understanding of apoptosis under thermal stress as well as the identification of potential targets to improve hyperthermia related treatment of lung cancer; the targeting of Ku70 might have therapeutic relevance in combination with siRNAs and/or specific HDACIs.

Supporting Information

Figure S1 Phase contrast photographs of H1299 cells two days after treatment with HDACIs (NAM at 20 mM or TsA at 300 nM final conc. or both for 4 h) then hyperthermia for 6 h. The image showed limited number of recovered cells as well as the rounded and floating apoptotic cells mainly in the colonies pre-treated with HDACIs then hyperthermia. HDACIs themselves did not induce significant cell death.
(TIF)

Figure S2 Hyperthermia-induced apoptosis was enhanced by HDACis. Pre-treatment of H1299 cells with DHACIs (NAM at 20 mM or TsA at 300 nM final conc. for four hours) significantly increased hyperthermia-induced apoptosis. Comparative cytograms show Annexin V staining after HDACis and hyperthermia in H1299 cells.
(TIF)

Figure S3 In PC-10 cells, Hyperthermia and HDACis combination-induced apoptosis was attenuated by Bax siRNA. PC-10 cells were either transfected with Bax siRNA or cont siRNA. When these cells were pre-treated with HDACIs (NAM at 20 mM or TsA at 300 nM final conc. for four hours) followed by hyperthermia (6 h), the apoptotic outcome was significantly decreased in the Bax KD cells compared with control ones. Comparative cytograms show Annexin V staining after HDACis and hyperthermia in Bax KD and control PC-10 cells.
(TIF)

Figure S4 Proteomic analysis of some apoptosis –related proteins in H1299 cells. Whole cell extracts were prepared after 0 h or 6 h hyperthermia (42.5°C). Bax, Ku70 and Bcl-2 were analyzed by immunoblotting. Six hours hyperthermia induced slight increase in Bax expression level and reduced Bcl-2 while Bcl-xL and Ku70 had not been affected. Analysis was performed in the same blot so each protein worked as a loading control for the other. A representative Coomassi Brilliant Blue (CBB) staining of the membrane was shown to act as a loading control.
(TIF)

Figure S5 Representative images show localization of Bcl-2 and Bax in lung cancer cell lines. Bax (green), Bcl-2 (green), and nuclei (red) were stained. Bax localization: cytosol in PC-10 cells (a) and in the cytoplasm and the nucleus in H1299 cells (b). Bcl-2 is localized in the cytoplasm in all cells tested (c,d).
(TIF)

Figure S6 Hyperthermia modulates Bax association with Ku70 in CHAPS buffer. PC-10 cells were incubated at 42.5°C for 0, 1, 3 and 6 h. then lysed in CHAPS buffer. Ku70 was co-immunoprecipitated from 2 mg total protein and Bax was detected in the immunoprecipitant by western analysis (upper panel). Total Ku70 levels showed no changes. Hyperthermia induced Bax up-regulation and enhanced association between Bax and Ku70. On the other hand, Bax was co-immunoprecipitated from similar cell lysates. Ku70 was detected in the immunoprecipitant. Again,after hyperthermia association between Ku70 and Bax was enhanced.
(TIF)

Figure S7 Hyperthermia did not change expression of HDAC6 or SirT-3. PC-10 was treated with hyperthermia (0–6 h) and cells were lysed and fractionated and blotted. Both HDAC-6 and SirT-3 expression was evaluated by immunoblotiing analysis. Immunodetection indicated that hyperthermia did not induce significant changes in the expression of both proteins in PC-10.
(TIF)

Figure S8 Ku70 is required for cytostatic arrest by hyperthermia. H1299 cells were transfected with Ku70-siRNA-2 or cont-siRNA-2 (100 nM) twice. 24 h after last transfection, equal cell numbers were subcultured for further 24 h, and then treated with hyperthermia for indicated time periods and then re-cultured at 37°C for 24 h (a) or 48 h (b) Cells were acquired by FACS analyzer for cell cycle analysis. A representative results is shown at each time point (24 h and 48 h).
(TIF)

Acknowledgments

Authors thank Prof. Shoichi Inoue and Dr. Takahiko Kobayashi for their technical advice.

Author Contributions

Conceived and designed the experiments: MH HW. Performed the experiments: MH AS.S ZM. Analyzed the data: MH AE.S YF. Contributed reagents/materials/analysis tools: YO NS. Wrote the paper: MH YO.

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