The expression of DAMP proteins HSP70 and cancer-testis antigen SPAG9 in peripheral blood of patients with HCC and lung cancer

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Abstract There are different views of how the immune system participates in the reaction to cancer. Here, we evaluated expression of DAMP proteins HSP70 and cancer-testis antigen SPAG9 in patients with hepatocellular carcinoma (HCC) and lung cancer to explore tumor immunity. Our analysis showed that levels of HSP70 and SPAG9 antibody were significantly higher in the serum of lung cancer and HCC patients than in the serum of healthy subjects ($P < 0.001$), but there were no differences in levels of HSP70 antibody in patients and controls. Levels of serum SPAG9 antibody in newly diagnosed lung cancer patients were significantly higher than in treated lung cancer patients ($P < 0.05$), but there were no differences in levels of HSP70 or HSP70 antibody. Levels of serum HSP70 and SPAG9 antibody, but not HSP70 antibody, were also higher in hepatitis/cirrhosis patients than in healthy subjects ($P = 0.005$, $P < 0.001$). Levels of serum SPAG9 antibody were significantly higher in HCC patients than in hepatitis/cirrhosis patients, but there were no differences in HSP70 or HSP70 antibody levels. Finally, levels of serum HSP70 and SPAG9 antibody were significantly higher in HCC patients than in lung cancer patients ($P < 0.05$, $P < 0.001$). These results indicate that cancer-testis antigen

SPAG9 induces a strong humoral immune response in cancer patients but HSP70 does not. These results show that SPAG9 has potential as a tumor-specific biomarker.

Keywords Heat shock protein 70 · Sperm-associated antigen 9 · Auto-antibody · Hepatocellular carcinoma · Lung cancer

Abbreviations

DAMPs	Damage-associated molecular patterns
HSP	Heat shock protein
PAMPs	Pathogen-associated molecular patterns
TLR	Toll-like receptor
APC	Antigen presenting cell
SNSD	Self/non-self discrimination
SPAG9	Sperm-associated antigen 9
HCC	Hepatocellular carcinoma
CTA	Cancer-testis antigen

Introduction

According to the “danger” theory of immunology, damage-associated molecular patterns (DAMPs) are released by the body’s cells as a signal that there is endogenous danger (Srikrishna and Freeze 2009). DAMPs, such as high mobility group protein B1 and heat shock proteins (HSPs), are released from damaged or necrotic tissue and by some activated immune cells (Pradeu and Cooper 2012). DAMPs and pathogen-associated molecular patterns (PAMPs) signal through toll-like receptors (TLRs) activate antigen presenting cells (APCs) and initiate the adaptive immune response (Miyaji et al. 2011). This theory is a challenge to the traditional self/non-self discrimination (SNSD) theory, which holds that activation of the immune system occurs strictly based on

In this paper, we discuss the how our findings relate to the self/non-self discrimination and danger theories of the involvement of the immune system in cancer development. We first put the DAMP protein and CTA combined research.

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Table 1 Comparison of gender and age between groups

Lung cancer	Control (n=54)	Newly diagnosed (n=45)	Treated (n=52)	Statistics	Statistical magnitude	P	Significance level
Age	57 ± 8	60 ± 11	57 ± 9	One-way ANOVA	F=1.466	0.234	0.05
Gender (m/f)	13/41	4/41	13/39	Chi-squared test	$\chi^2=4.867$	0.088	0.05
HCC	Control	Hepatitis/cirrhosis (n=41)	HCC (n=31)	Statistics	Statistical magnitude	P	Significance level
Age	57 ± 8	55 ± 9	56 ± 11	Kruskal-Wallis <i>H</i> test	$\chi^2=1.294$	0.524	0.05
Gender (m/f)	13/41	7/34	5/26	Chi-squared test	$\chi^2=1.074$	0.584	0.05

whether or not an entity is foreign. The danger theory, in contrast, posits that the immune system is only triggered by “dangerous” entities.

Extracellular HSP70 and SPAG9 are proteins released by tumor cells (Li et al. 2013; Sinha et al. 2013a), but their characteristics and the roles played in the development of cancer are different. HSP70 is produced in response to internal and external stresses such as trauma, infection, and cancer (Radons 2016; Ren et al. 2016a). HSP70 has a dual role: When present inside cells, it can induce resistance to apoptosis; outside the cell, it can promote apoptosis (Lanneau et al. 2007). Extracellular HSP70 as an endogenous DAMP protein participates in the innate immune response and in the adaptive immune response (Chen and Cao 2010). The ability of HSP70 to accelerate the elimination of tumor cells has been the focus of considerable research (Kumar et al. 2016; Murphy 2013; Meng et al. 2011).

SPAG9 is a member of the cancer-testis antigen (CTA) family, which, as a scaffold protein, plays an important role in the process of sperm-egg fusion (Jagadish et al. 2005a; Jagadish et al. 2005b). In addition to expression in germ cells, it is also expressed in a variety of tumor tissues and can induce a strong humoral immune response indicated by production of auto-antibodies (Garg et al. 2007; Agarwal et al. 2014a; Kanojia et al. 2013; Xie et al. 2014; Ren et al. 2016b). That SPAG9 can induce an immune response in the context of danger (that is, cancer) supports the SNSD theory.

Like SPAG9, HSP70 levels are upregulated in certain cancer patients (Luk et al. 2006; Alaiya et al. 2001). In this study, levels of HSP70, HSP70 antibodies, and SPAG9 antibodies were compared in peripheral blood of patients with lung cancer and hepatocellular carcinoma (HCC) and healthy subjects. We compared the similarities and differences

between expression profiles of antigens and auto-antibodies and evaluate the value of these markers in the diagnosis of lung cancer and HCC. We also speculate on how immune responses to these two proteins support the SNSD and danger theories of the immune response to cancer.

Material and methods

Patients and sample collection

Patients were treated in the Tumor Hospital and Second People's Hospital of Hunan Province. Diagnoses were confirmed by a pathologist. Patients consented to specimen collection, and the study was approved by the ethics committee of Second People's Hospital of Hunan Province. Serum samples were obtained from 97 lung cancer patients; the 45 patients who had not yet received treatment were classified as the newly diagnosed group. The 52 lung cancer patients who had received radiotherapy and chemotherapy were classified as the treated group. Lung cancer cases were diagnosed by CT, MRI, fiber-optic bronchoscopy, and percutaneous lung biopsy.

Samples from HCC patients with cancers caused by HBV or by HCV who had not received any treatment and 41 patients with hepatitis/cirrhosis caused by HBV or HCV without HCC were analyzed. HCC was diagnosed as previously reported (Song et al. 2013). HCC inclusion criteria were no prior anti-cancer treatment (drug, radiotherapy, or chemotherapy) and HBV or HCV positive or both positive. Exclusion criteria were pregnancy, reproductive system embryonic tumors, or metastatic liver cancer.

Fig. 1 The means of (a) the signal intensities in the SPAG9 ELISA, (b) the concentrations of serum HSP70, and (c) the serum HSP70 antibody based on ELISA in lung cancer and HCC patients and controls,

Fifty-four healthy subjects were recruited from the Health Screening Center of Hunan Second People's Hospital. There were no significant differences in age or gender among groups. Characteristics of the patients are shown in Table 1. Peripheral blood was collected, and the serum was separated by centrifuging blood at $1000\times g$ for 10 min. The serum was stored at -80°C until analysis.

ELISA

Recombinant human SPAG9 protein (r-hSPAG9, Abnova) was used as antigen in an ELISA to detect serum anti-SPAG9 IgG antibody levels (Kanojia et al. 2009). Basal levels in the ELISA were established using the serum from 35 healthy donors, and the cutoff signal intensity (mean $\pm$ 1.96 SD) was an OD of 0.416 (0.187 ± 0.229). The absorbance was read at 450 nm with 630 nm as reference filter, and the intra-assay and inter-assay coefficients of variation were 2.3 and 8.6%, respectively. HSP70 quantitative analysis was performed on the Multiskan MK3 automatic enzyme immunoassay instrument from Labsystems with reagents from Adlitteram Diagnostic Laboratories. HSP70 quantitative analysis was performed on the Labsystems Multiskan MK3 with reagents from Adlitteram Diagnostic Laboratories.

Statistical analyses

The Pearson chi-squared test, Fisher's exact test, Student's *t* test for unpaired data, Wilcoxon signed-rank test, Mann-Whitney *U* test, and Kruskal-Wallis test were performed using the SPSS 16.0 statistical software. Results are expressed as means $\pm$ standard deviations (SD). All *P* values were two-sided, and a *P* value <0.05 was considered statistically significant.

Results

Levels of serum SPAG9 antibody, HSP70, and HSP70 antibody in lung cancer and HCC patients

The means of the signal intensities in the SPAG9 ELISA for lung cancer patients (0.579 ± 0.472) and HCC patients (0.590 ± 0.274) were significantly higher than in healthy subjects (0.187 ± 0.117) ($P < 0.001$), and there were statistically significant differences between the two types of tumors. The concentrations of serum HSP70 in sera from lung cancer patients (13.26 ± 9.37) and HCC patients (23.23 ± 13.64) were significantly higher than in healthy

Table 2 Comparison of serum HSP70 levels between HCC and lung cancer groups

	Control (<i>n</i> =54)	Lung cancer (<i>n</i> =97)	HCC (<i>n</i> =31)	Statistics	Statistical magnitude	<i>P</i>	Significance level
Age	57 $\pm$ 8	58 $\pm$ 10	56 $\pm$ 11	One-way ANOVA	$F=0.728$	0.484	0.05
Gender (m/f)	13/41	17/80	5/26	Kruskal-Wallis <i>H</i> test	$\chi^2=1.189$	0.552	0.05
HSP70				Kruskal-Wallis <i>H</i> test	$\chi^2=38.653$	<0.001	0.05
Multiple comparison				Mann-Whitney <i>U</i> test and corrected with Bonferroni		<0.001	0.0167
						<0.001	
						<0.001

Fig. 2 Levels of (a) serum SPAG9 antibody, (b) serum HSP70, and (c) serum HSP70 antibody in newly diagnosed lung cancer patients, treated lung cancer patients, and controls

subjects ($P < 0.001$) irrespective of the disease phase. The result that the concentration of SPAG9 in lung cancer patient sera was lower than that in HCC patient sera was statistically significant ($P < 0.05$). There were no differences in the levels of serum HSP70 antibody among the patients with HCC or lung cancer and healthy controls (Fig. 1 and Table 2).

Levels of serum SPAG9 antibody, HSP70, and HSP70 antibody in patients newly diagnosed with lung cancer and treated for lung cancer

The means of the signal intensity in the SPAG9 ELISA for newly diagnosed lung cancer patients (0.579 ± 0.472) and treated lung cancer patients (0.357 ± 0.225) were significantly higher than in

Table 3 Comparison of serum HSP70 and SPAG9 antibody levels in different groups

Lung cancer	Control	Newly diagnosed	Treated	Statistics	Statistical magnitude	<i>P</i>	Significance level
SPAG9 Ab				Kruskal-Wallis <i>H</i> test	$\chi^2=33.760$	<0.001	0.05
Multiple comparison				Mann-Whitney <i>U</i> test and corrected with Bonferroni		<0.001	0.0167
						<0.001	
						0.005	
HSP70				Kruskal-Wallis <i>H</i> test	$\chi^2=14.204$	0.001	0.05
Multiple comparison				Mann-Whitney <i>U</i> test and corrected with Bonferroni		0.003	0.0167
						<0.001	
						0.853	
HSP70 Ab				Kruskal-Wallis <i>H</i> test	$\chi^2=1.233$	0.540	0.05
HCC	Control	Hepatitis / cirrhosis	HCC	Statistics	Statistical magnitude	<i>P</i>	Significance level
SPAG9 Ab				Kruskal-Wallis <i>H</i> test	$\chi^2=39.136$	<0.001	0.05
Multiple comparison				Mann-Whitney <i>U</i> test and corrected with Bonferroni		0.003	0.0167
						<0.001	
						<0.001	
HSP70				Kruskal-Wallis <i>H</i> test	$\chi^2=42.322$	<0.001	0.05
Multiple comparison				Mann-Whitney <i>U</i> test and corrected with Bonferroni		<0.001	0.0167
						<0.001	
						0.442	
HSP70 Ab				Kruskal-Wallis <i>H</i> test	$\chi^2=5.646$	0.059	0.05

Fig. 3 Levels of (a) serum SPAG9 antibody, (b) serum HSP70, and (c) serum HSP70 antibody in HCC patients, hepatitis/cirrhosis patients, and healthy controls

healthy subjects (0.187 ± 0.117) ($P < 0.001$). The mean of the signal intensity in the SPAG9 ELISA for newly diagnosed lung cancer patients was also significantly higher than in treated lung cancer patients ($P = 0.005$). Levels of serum HSP70 in newly diagnosed lung cancer patients (13.26 ± 9.37) and treated lung cancer patients (11.88 ± 5.92) were significantly higher than in healthy subjects (8.55 ± 3.53) ($P = 0.003$, $P < 0.001$), but there was no statistical difference between newly diagnosed lung cancer patients and treated lung cancer patients. There were no differences in the levels of serum HSP70 antibody among the patients newly diagnosed with lung cancer and treated for lung cancer and healthy controls (Fig. 2 and Table 3).

Levels of serum SPAG9 antibody, HSP70, and HSP70 antibody in HCC and hepatitis/cirrhosis patients

The means of the signal intensities in the SPAG9 ELISA for HCC patients of (0.590 ± 0.274) and hepatitis/cirrhosis patients (0.445 ± 0.515) were significantly higher than that in healthy subjects (0.187 ± 0.117) ($P < 0.001$ and $P = 0.003$, respectively). The mean of the signal intensity in the SPAG9 ELISA for HCC

patients was significantly higher than that for hepatitis/cirrhosis patients ($P < 0.001$). Levels of serum HSP70 in HCC patients (23.23 ± 13.64) and in hepatitis/cirrhosis patients (22.14 ± 14.10) were significantly higher than in healthy subjects (8.55 ± 3.53) ($P < 0.001$) but there was no statistical difference between HCC and hepatitis/cirrhosis patients. There were no differences in the levels of serum HSP70 antibody among the patients with HCC and hepatitis/cirrhosis and healthy controls. (Fig. 3 and Table 3).

Discussion

The self/non-self discrimination theory maintains that the body distinguishes between self and non-self components (Bretscher and Cohn 1970; Burnet 1959). In 1994, Matzinger (1994) put forward the danger theory that holds that dangerous situations rather than simply non-self antigen can activate the immune response. During infection, stress, injury, necrosis, or tumor formation, for example, certain molecules can trigger host defenses and upregulate costimulatory

Fig. 4 Mechanism of SPAG9 antibody production that, based on SNSD theory, activates the Th1 arm of the immune system

Fig. 5 Tumor cells release DAMPs, such as HSP70 and SPAG9, which induce the Th2 humoral immune response as predicted by the danger theory

molecules that induce dendritic cell maturation, leading to effective antigen presentation to T and B cells.

Endogenous proteins such as SPAG9 can induce strong humoral immune responses (Wang et al. 2013; Yu et al. 2012; Agarwal et al. 2014b) when present on the surface of the tumor cells or released from tumor cells to the outside of the cell (Sinha et al. 2013b). In this research, we found that the levels of serum SPAG9 antibody in lung cancer patients and HCC patients were significantly higher than in healthy subjects. The immune response to SPAG9 can be categorized as

non-self antigen activation of the immune response because in these cancer patients, SPAG9 is present in peripheral blood, a location where it is not normally found, because of the characteristics of CTA (Fig. 4).

In support of the danger theory, however, HSP70 and SPAG9 are DAMPs and serve as danger signals to initiate the humoral immune response (Fig. 5). Interestingly, our data showed that unlike SPAG9, which stimulated the immune system to produce antibodies both in HCC and lung cancer patients, the elevated

Fig. 6 HSP70 binds to TLR4 triggering tumor self-protection mechanisms leading to tumor cell secretion of SPAG9

levels of HSP70 in the serum from cancer patients were not accompanied by increases in levels of antibody to this protein.

TLRs, especially TLR4, recognize distinct PAMPs and DAMPs that play critical roles in both innate and adaptive immunity. Studies have found that TLRs are expressed on the surfaces of a variety of immune cells and on the surfaces of tumor cells (Huang et al. 2008). The TLR signaling pathways activate ERK, JNK, and p38 (Takeda and Akira 2004), which in turn regulate many immunologically relevant proteins that can trigger tumor self-protection mechanisms (Huang et al. 2005). In this study, we found that on average the serum levels of HSP70 and of SPAG9 antibody were significantly higher in patients with lung cancer and HCC than in healthy controls. No differences in HSP70 antibody levels were observed. It may be that HSP70 released by tumor cells binds to receptors (such as TLR4) on tumor cell surfaces to promote the expression of tumor-specific proteins (such as SPAG9) that in turn promote the growth and metastasis of tumor cells (Fig. 6).

The level of serum SPAG9 antibody in newly diagnosed lung cancer patients was significantly higher than in treated lung cancer patients, but there were no differences in levels of HSP70 or HSP70 antibody. Similarly, levels of serum SPAG9 antibody in HCC patients were significantly higher than in hepatitis/cirrhosis patients but there were no differences in levels of HSP70 or HSP70 antibody. This indicated that HSP70, unlike SPAG9, stimulates the body's immune system to produce specific antibodies. Concentrations of HSP70 and of SPAG9 antibody in patients with HCC were significantly higher than in patients with lung cancer; the reason is not clear but is worthy of further research. Each of the two theories of how the immune system is activated, the SNSD theory and the danger theory, has some support from this study. The immune stimulatory mechanisms of the human body are very complicated, and we have a long way to go to understand the truth.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement The study was discussed by the ethics committee of our institute and did not involve the interests of patients; approval was given by the committee. All subjects signed informed consent agreements.

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