

CLINICAL STUDY

IL-6 mediated JAK/STAT3 signaling pathway in cancer patients with cachexia

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ABSTRACT

OBJECTIVES: We investigated the changes in the IL-6 and STAT3 expression levels in cachectic and non-cachectic patients with gastric, lung and breast cancer and evaluated the association between IL-6 and STAT3 levels and cancer types in terms of cachexia condition.

BACKGROUND: Cancer-associated cachexia, observed in nearly 50–80 % of cancer patients, has drawn attention in advanced patients. IL-6/JAK/STAT pathway plays an essential role in the progression of cancer cachexia through the regulation of the inflammatory response.

METHODS: This study consisted of 48 gastric, breast and lung cancer patients (18 cachectic and 30 non-cachectic) and healthy individuals. Total RNA isolation and cDNA synthesis was performed after the collection of blood samples. IL-6 and STAT3 expression levels were analyzed by RT-PCR analysis.

RESULTS: Our findings demonstrated that IL-6 mRNA levels considerably increased 19.89 ± 8.25 , 5.18 ± 2.81 and 15.33 ± 9.54 -fold in gastric, lung and breast cancer patients with cachexia, respectively. Additionally, a 16.67 ± 7.13 , 14.21 ± 11.72 and 8.85 ± 3.89 -fold increase in the STAT3 expression level was detected in cachectic gastric, lung and breast cancer patients, respectively ($p < 0.01$).

CONCLUSION: STAT3 may be considered as a therapeutic target for cachectic patients with gastric, lung and breast cancer. Furthermore, IL-6 mediates STAT3 activation in cachectic gastric and breast cancer patients (Tab. 5, Fig. 2, Ref. 62). Text in PDF www.elis.sk.

KEY WORDS: cachexia, IL-6, STAT3, gastric cancer, lung cancer, breast cancer.

Introduction

Cachexia, which accounts for 20 % cancer deaths, is a multifactorial syndrome and defined by weight loss $> 5\%$ or $> 2\%$ with a body mass index (BMI) $< 20 \text{ kg/m}^2$ over 6 months and the loss of skeletal muscle ($< 7.26 \text{ kg/m}^2$ for males and $< 5.45 \text{ kg/m}^2$ for females), adipose tissue and skeletal muscle mass. Therefore, cachexia is associated with a poor prognosis, toxicities, reduces overall survival and thus, cachexia has a negative impact on the quality of life in cancer patients. Furthermore, no beneficial effects are observed with parenteral nutrition and supplementation to treat cachectic cancer patients due to anorexia, anemia and systemic inflammation (1–6).

The interleukin 6 (IL-6) mediated Janus Kinase (JAK)/ Signal transducer and activator of transcription 3 (STAT3) pathway play an important role in cancer cachexia through induction of systemic

inflammatory response (7, 8). STAT3 is a transcription factor and aberrant activation of the phosphorylated STAT3 (pSTAT3) is involved in nearly 70 % of human cancers due to promoting tumor growth through regulating multiple genes and/or pathways (9, 10). Interleukin 6 (IL-6) is an interleukin that mediates the immune response, the acute phase response and inflammation through its receptors (IL-6 receptor (IL-6R) and gp130). IL-6 is highly up-regulated during inflammation in response to tissue injury or stress and other pro-inflammatory cytokines (11–13). JAK/STAT is activated by the phosphorylation of IL-6 tyrosine residues in the cytoplasmic domain of gp130. Thus, IL-6 plays a crucial role in the inflammation-mediated “cancers types”, including prostate, breast, gastric, colon, lung and brain due to inducing different cytoplasmic tyrosine kinases and transcription factors and thus, overexpression of IL-6 is associated with poor prognosis in many cancers. Previous studies mentioned that IL-6 mediated STAT3 activation could be a therapeutic target in gastric, glioblastoma, breast, ovarian and lung cancer patients due to mediating muscle wasting (14–20). However, further studies are still needed to clarify the association between the changes in IL-6 and STAT-3 levels and cancer patients with and without cachexia.

For this purpose, we for the first time investigated the expression level of IL-6 and STAT3 in cachectic and non-cachectic cancer patients levels with gastric, lung and breast cancer in comparison to the control groups, in order to identify the effect of IL-6 signaling

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Tab. 1. Primer sequences.

Gene Name	Direction	Primer sequence (5'-3')
STAT3	Forward	ATCACGCCTTCTACAGACTGC
STAT3	Reverse	CATCCTGGAGATTCTTACCACT
IL-6	Forward	AACCTGAACCTTCCAAAGATGG
IL-6	Reverse	TCTGGCTTGTTCCTCACTACT

Tab. 2. Clinical data of cancer patients with cachexia and without cachexia and healthy control subjects.

	Cachectic cancer patients (n=18)	Non-cachectic cancer patients (n=30)	Control (n=25)
Age (years)	55.28 ±9.55	61.80 ±10.02	54.40±8.3
Gender (M/F)	9 / 9	16 / 14	13/12
Tumor Types	Gastric Cancer (n=5) Invasive Ductal Breast Cancer (n=6) Lung Cancer (n=7)	Gastric Cancer (n=10) Invasive Ductal Breast Cancer (n=10) Lung Cancer (n=10)	
Grade			
1	6.3%	22.7%	
2	25.0%	31.8%	
3	68.8%	45.5%	
T Stage			
T1	23.5%	31.0%	
T2	11.8%	20.7%	
T3	29.4%	20.7%	
T4	35.3%	27.6%	
N stage			
N0	16.7%	14.3%	
N1	27.8%	25.0%	
N2	33.3%	35.7%	
N3	22.2%	25.0%	
M stage			
M0	33.3%	46.7%	
M1	66.7%	53.3%	
Weight (kg)	56.35±6.03	76.10±12.32	84.42±5.02
BMI (kg/m ²)	17.48±9.79	28.85±7.31	28.11±5.1
Total serum protein (g/dL)	6.87±0.58	6.66±1.54	7.4±0.80
Albumin (g/dL)	3.7±9.52	2.3±1.85	4.2±0.30

on STAT3 activation in cancer cachexia. Furthermore, we explored the association between IL-6 mediated the activation of STAT3 and gastric, lung and breast cancer patients with and without cachexia.

Materials and methods

Patient population

This study consisted of forty-eight patients (n = 48) with gastric, breast and lung cancer and the patients, were admitted to Sakarya University Faculty of Medicine Department of Oncology before any surgical or medical procedures. Patients, who had received prior treatment before admission were excluded. Eighteen of the patients (n = 18) presented with cachexia signs and symptoms during the study period and were diagnosed with cachexia by corresponding oncology physicians according to diagnostic criteria for cachexia. Thirty (n = 30) of the patients did not show cachexia symptomatology and maintained a higher BMI than cachectic cancer patients during the study period. The patients were

categorized into six groups: cachectic patients with gastric (n = 5), lung (n = 7) and breast (n = 6) cancer and non-cachectic patients with gastric (n = 10), lung cancer (n = 10) and breast cancer (n = 10) compared with healthy individuals as controls (n = 25). This study was approved by the Ethics Committee of Sakarya University (Application number: 16214662/050.01.04/23) and written consent was obtained from all the patients included in this study.

Total RNA isolation and cDNA synthesis

Blood samples from patients were collected and stored at -80 °C for RNA isolation. Total RNA was isolated by Purelink RNA Mini Kit (Invitrogen, Cat. 12183018A) according to the manufacturer's instructions. The concentration of total RNA was analyzed by Qubit RNA Assay Kit and Qubit 4 Fluorometer (Invitrogen/Molecular Probes). Then, 100 ng of total RNA was reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems).

Real-time PCR (RT-PCR) analysis

In order to analyze the changes in IL-6 and STAT3 expression levels, RT-PCR was performed in 96-well optical reaction plates with a StepOnePlus Real-Time PCR Systems (v. 2.0, Applied Biosystems) by SYBR Green mix. ACTB was used for the reference gene. Primer sequences of IL-6 and STAT3 were shown in Table 1.

Statistical analysis

Statistical analysis was performed by the SPSS software package, version 21.0 (SPSS Inc. USA). All data were expressed as the mean ± standard deviation (SD) and One-Way ANOVA analysis with Post-Tukey test was performed for multiple comparison. Univariate ANOVA analysis was used to determine the interaction IL-6 and STAT3 levels with cancer types in terms of cancer cachexia. The percent difference was calculated by Δ% formula (21). The fold change of the IL-6 and STAT3 levels was statistically analyzed by software (<https://www.qiagen.com/tr/shop/genes-and-pathways/data-analysis-center-overview-page/other-real-time-pcr-probes-or-primers-data-analysis-center/>). Statistical significance was set at p < 0.05.

Results

Patients characteristic

From April 2017 to April 2018, 48 cancer patients (18 cachectic and 30 non-cachectic) and 25 healthy controls were included in this study and clinical characteristic of cachectic and non-cachectic cancer patients included to the study were presented in Tables 2 and 3. In terms of age, the cachectic and non-cachectic cancer patients in each cancer type (gastric, lung and breast cancer patients) and controls were similar. The average age of gastric, lung and breast cachectic cancer patients was 61.80 ± 10.04, 59.00 ± 3.46 and 45.50 ± 6.22 (mean ± SD), respectively compared with non-cachectic patients with gastric, lung and breast cancer (65.90 ± 9.62, 65.40 ± 5.56 and 54.10 ± 10.4, respectively). BMI was significantly lower in cachectic cancer patients than in non-cachectic patients and the control. However, cachectic gastric and lung cancer patients indicated a significantly less BMI values than breast cancer patients.

Tab. 3. Characteristics of patients with gastric, lung and breast cancer with cachexia and without cachexia.

	Cachectic			Non-Cachectic		
	Gastric cancer patients (n=5)	Lung cancer patients (n=7)	Breast cancer patients (n=6)	Gastric cancer patients (n=10)	Lung cancer patients (n=10)	Breast cancer patients (n=10)
Age (years)	61.80±10.04	59.00±3.46	45.50±6.22	65.90±9.62	65.40±5.56	54.10±10.4
Gender (M/F)	5/0	4/3	0/6	6/4	10/0	0/10
Grade						
1	0.0%	0.0%	16.7%	14.3%	0.0%	40.0%
2	20.0%	0.0%	50.0%	28.6%	20.0%	40.0%
3	80.0%	100.0%	33.3%	57.1%	80.0%	20.0%
T Stage						
T1	0.0%	0.0%	66.7%	22.2%	0.0%	70.0%
T2	0.0%	16.7%	16.7%	11.1%	30.0%	20.0%
T3	80.0%	16.7%	0.0%	33.3%	20.0%	10.0%
T4	20.0%	66.7%	16.7%	33.3%	50.0%	0.0%
N stage						
N0	20.0%	14.3%	16.7%	12.5%	0.0%	30.0%
N1	20.0%	0.0%	66.7%	12.5%	10.0%	50.0%
N2	0.0%	71.4%	16.7%	12.5%	40.0%	20.0%
N3	60.0%	14.3%	0.0%	62.5%	50.0%	
M stage						
M0	0.0%	14.35%	83.3%	60.0%	20.0%	60.0%
M1	100%	85.7%	16.7%	40.0%	80.0%	40.0%
Weight (kg)	54.50±4.04	55.29±6.73	58.83±7.44	69.20±9.26	76.5±4.10	83.00±11.71
BMI (kg/m ²)	18.90±1.36	19.84±5.64	24.48±3.82	23.80±3.41	25.50±1.82	33.90±6.70
Total serum Protein (g/dL)	6.60±0.1	6.64±0.59	7.43±0.35	5.6±2.29	7.08±2.37	7.23±0.51
Albumin (g/dL)	3.28±0.53	3.85±0.06	4.2±0.2	2.06±1.84	2.37±1.70	22.47±2.16

IL-6 and STAT3 expression levels in cancer patients with or without cachexia

The expression levels of IL-6 and STAT3 were analyzed in gastric (n = 5), lung (n = 7) and breast cancer (n = 6) with cachexia compared with non-cachectic gastric (n = 10), lung (n = 10) and breast cancer patients (n = 10) and healthy group (n = 25) as indicated in Figure 1. In gastric, lung and breast cancer patients with cachexia, IL-6 mRNA levels remarkably increased 19.89 ± 8.25, 5.18 ± 2.81 and 15.33 ± 9.54-fold, respectively (p < 0.01) compared to the control group (0.67 ± 0.50). Additionally, a 4.48 ± 2.10 (p = 0.004) and 4.62 ± 3.53-fold (p = 0.026) increase in IL-6 expression level was observed in gastric and breast cancer patients without cachexia. However, the expression of IL-6 was higher in non-cachectic lung cancer patients (8.03 ± 4.38) than in cachectic patients (5.18 ± 2.81; p < 0.01). Furthermore, the STAT3 expression level was significantly higher in gastric (16.67 ± 7.13), lung (14.21 ± 11.72) and breast cancer (8.85 ± 3.89) patients with cachexia (p < 0.01) compared to the control group (0.78 ± 0.40). In cancer patients without cachexia, STAT3 expression level increased by 1.72 ± 0.72 (p = 0.587), 5.83 ± 4.99 (p = 0.037) and 2.03 ± 0.88-fold (p = 0.102) in gastric, lung and breast cancer patients, respectively.

The association between IL-6 and STAT3 expression levels and cancer types

We compared the association between IL-6 and STAT3 expression levels and patients with cachexia and without cachexia in terms of cancer type (gastric, lung and breast). In Figure 2, the

changes in the expression level of IL-6 was significant between gastric and lung cancer patients (p = 0.009) with cachexia. Additionally, there was a significant difference between gastric and lung cancer patients (p = 0.012) or lung and breast cancer patients (p = 0.021) without cachexia according to STAT3 expression level. However, there was no significant difference in STAT3 as well as IL-6 levels among cachectic cancer patients.

We performed univariate ANOVA analysis to statistically compare cachectic and non-cachectic cancer patients in terms of cancer type and IL-6 and STAT3 expression levels. In Table 4, all the measured values of IL-6 expression level were statistically different in cachectic and non-cachectic gastric, lung and breast cancer patients (p < 0.05). Besides, the interaction between cancer type and cachexia status was statistically significant (F = 13.127; p = 0.001). Thus, IL-6 mRNA level was higher in cachectic lung and breast cancer patients than in non-cachectic cancer patients. When the ratio of percentage differences in the IL-6 level was evaluated between cachectic and non-cachectic patients, there was 79.07 %, and 69.86 % increase in gastric and breast cancer patients, respectively. However, the expression of IL-6 decreased by -55.01 % in lung cancer patients with cachexia. When the ratio of percentage differences in the IL-6 level among the types of cancer was examined, there was -40.09 % -11.22 % and 20.60 % differences between the lung and gastric cancer patients, the breast and gastric cancer patients and the breast and lung cancer patients, respectively.

Furthermore, all the measured values of STAT3 mRNA level were statistically different in gastric, lung and breast cancer pa-

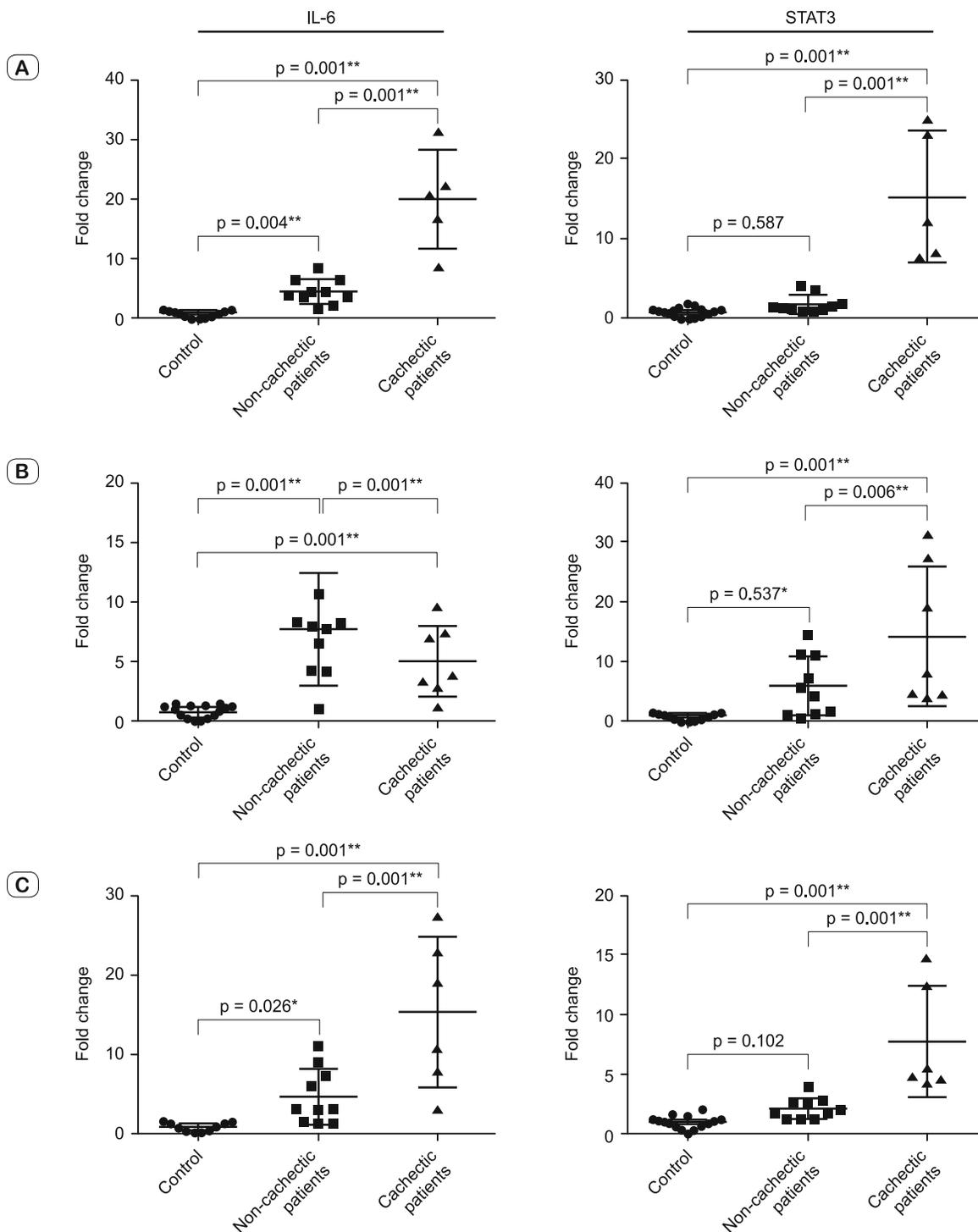


Fig. 1. The mRNA level of IL-6 and STAT3 in cachectic and non-cachectic cancer patients compared to the healthy subjects. (A) Gastric, (B) Lung and (C) Breast cancer patients (* $p < 0.05$, ** $p < 0.01$).

tients in Table 5 ($p < 0.05$). Additionally, the expression of STAT3 was statistically different according to the cachectic and non-cachectic status ($p < 0.01$). The interaction between the cancer type and cachexia status was statistically significant ($F = 3.204$; $p = 0.047$). Therefore, STAT3 expression level was higher in cancer

patients with cachexia than without cachexia. When the ratio of percentage differences in the STAT3 level between cachectic and non-cachectic patients were investigated, there was 89.68 %, 58.98 % and 77.06 % increase in the STAT3 levels of gastric, lung and breast cancer patients with cachexia, respectively compared with

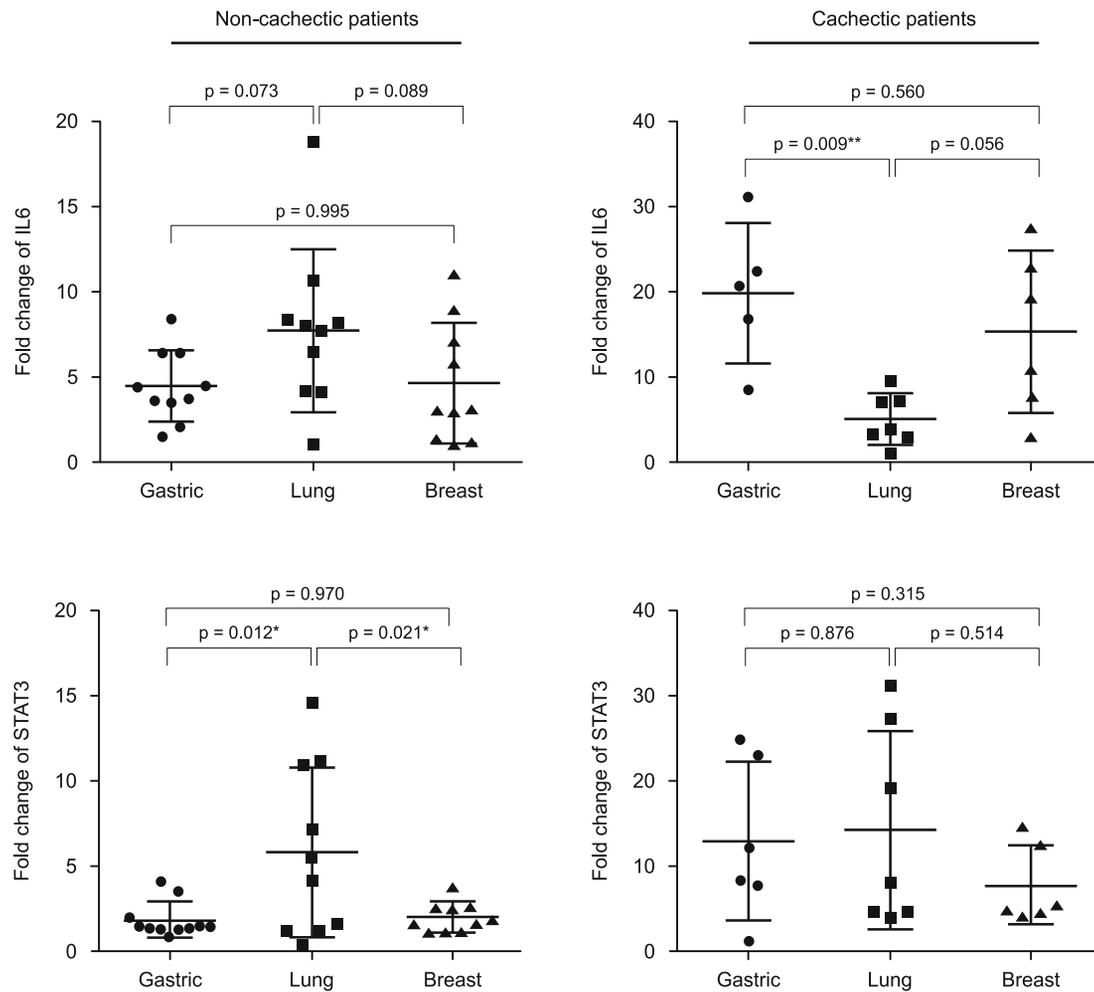


Fig. 2. Comparison of IL-6 and STAT3 level in gastric, lung and breast cancer patients according to cachectic and non-cachectic condition (*p < 0.05, **p < 0.01).

non-cachectic groups. When the ratio of percentage differences in the STAT3 level among the types of cancer were examined, there was 27.69 %, -46.19 % and -102.18 % differences between the lung and gastric cancer patients, the breast and gastric cancer patients and the breast and lung cancer patients, respectively.

Discussion

Cachexia is a multifactorial syndrome observed in advanced-stage cancer patients, which has a considerable impact on patient prognosis and is a major cause of mortality (1–6). As the molecular mechanisms underlying cachexia have not been fully elucidated yet, new molecular biomarkers of cancer cachexia are urgently explored. In the literature, single nucleotide polymorphism in different genes and cytokines, differentially expressed alternatively spliced genes (DASGs), some cytokine (IL-6, and TNF- α) serum levels and epigenetic mechanisms have been associated with cachexia in patients with gastric, head and neck, pancreatic and lung cancer (22–32). Additionally, recent studies have indicated that high systemic IL-6 levels are linked to worse prognosis in different

cancer patients (gastric, non-small lung, pancreas etc.) (33–35). However, no prior studies have examined the association IL-6-mediated STAT3 activation between cancer patients with cancer cachexia, to our knowledge. In the present study, we for the first time investigated the association between the expression levels of IL-6 and STAT3 in patients with cachectic and non-cachectic gastric, lung and breast cancer. We found that IL-6 mRNA level was significantly higher in cachectic gastric and breast cancer patients than non-cachectic patients ($p < 0.01$). Additionally, STAT-3 level was remarkably higher in all types of cancer patients with cachexia at any TNM stage than non-cachectic patients ($p < 0.01$). However, cachectic lung cancer patients indicated lower IL-6 mRNA levels than patients without cachexia. Furthermore, there was a significant interaction between IL-6 and STAT-3 mRNA level and cancer types in terms of cancer cachexia.

According to an epidemiological analysis of cachexia in patients with advanced cancer ($n = 140$), the prevalence of cachexia in gastric cancer patients was determined as 76.5 % (36). Inflammation is one of the most important cause in the development of gastric cancer. In the inflammatory process, different cytokines

Tab. 4. Comparison of IL-6 expression level in cachectic and non-cachectic cancer patients.

Grup&Cancer Types	N	Cachectic patients	Non- Cachectic patients	%Δ	Total	F	p	
		(n=18)	(n=30)		(n=48)			
		$\bar{X}\pm SD$	$\bar{X}\pm SD$					
Gastric (X ₁)	15	19.89±8.25	4.48±2.10	79.07	9.61±8.88 ^a	4.518	0.017*	
Lung (X ₂)	17	5.18±2.81	8.03±4.38	-55.01	6.86±3.98 ^b			
Breast (X ₃)	16	15.33±9.54	4.62±3.53	69.86	8.64±8.15 ^{ab}			
Total	48	12.65±9.30	5.71±3.74		8.31±7.17			Grup X Cancer Types Interaction
					F=25.534; p=0.001**		F=13.127; p=0.001**	
%Δ (X ₂ -X ₁)						-40.09		
%Δ (X ₃ -X ₁)						-11.22		
%Δ (X ₃ -X ₂)						20.60		

*p < 0.05; **p < 0.01; a,b: Different letters represent differences between groups; \bar{X} : Mean; SD: Standard Deviation; IL-6 value for the control group: 0.67±0.50; %Δ: (X₂-X₁)/X₂*100

Tab. 5. Comparison of STAT3 expression level in cachectic and non-cachectic cancer patients.

Grup & Cancer Types	N	Cachectic patients	Non- Cachectic patients	%Δ	Total	F	p	
		(n=18)	(n=30)		(n=48)			
		$\bar{X}\pm SD$	$\bar{X}\pm SD$					
Gastric (X ₁)	15	16.67±7.13	1.72±0.72	89.68	6.71±8.25 ^{ab}	4.473	0.015*	
Lung (X ₂)	17	14.21±11.72	5.83±4.99	58.98	9.28±9.14 ^a			
Breast (X ₃)	16	8.85±3.89	2.03±0.88	77.06	4.59±4.14 ^b			
Total	48	13.11±8.69	3.19±3.43		4.81±6.82			Grup X Cancer Types Interaction
					F=55.210; p=0.001**		F=3.204; p=0.047*	
%Δ (X ₂ -X ₁)						27.69		
%Δ (X ₃ -X ₁)						-46.19		
%Δ (X ₃ -X ₂)						-102.18		

*p < 0.05; **p < 0.01; a,b: Different letters represent differences between groups; \bar{X} : Mean; SD: Standard Deviation; STAT3 value for the control group: 0.78±0.40; %Δ: (X₂-X₁)/X₂*100

such as TNF alpha, IL-1, IL-6, IL-10 induce the activation of intracellular signaling pathways including PI3K/AKT and JAK/STAT (37–40). Additionally, Helicobacter pylori infection is linked to an increased risk of developing gastric cancer due to alteration the expression level of IL-6 (41, 42). Furthermore, some studies have also showed that gastric cancer patients with liver metastases have higher levels of IL-6 compared to the healthy controls and high serum IL-6 level is associated with tumor invasion, tumor size and lymph node metastasis and IL-6 mediated STAT3 activation plays a crucial role in increasing tumor size and proliferation of gastric cancer (43–48). Our results demonstrated that IL-6 and STAT3 expression level were considerably higher in cachectic gastric cancer patients levels compared to the non-cachectic and healthy control group (p < 0.01). Therefore, our findings suggest that high IL-6 and STAT3 mRNA level is associated with the development of cachexia and poor prognosis in gastric cancer patients.

In the literature, 45.6 % of patients receiving chemotherapy with advanced lung cancer present cancer cachexia signs and symptoms. Therefore, cachexia is commonly prevalent in particularly lung and gastrointestinal cancer patients (49). In the study of patients with different stages of lung cancer, IL-6 level was up-regulated compared with healthy subjects (33). Additionally, Miller et al (2017) state that IL-6 induces hyperactivation of STAT3 transcription factor via IL-6 cytokine family signaling receptor, gp130, and IL-6 regulated STAT3 activation results in cachexia during Kras-driven lung carcinogenesis, *in vivo* (18). In the present study,

we found that the expression level of IL-6 was higher in cachectic and non-cachectic lung cancer patients than in the healthy controls (p < 0.05). Interestingly, the mRNA level of IL-6 was lower in lung cancer patients with cachexia than that in patients without cachexia. According to these findings, the downregulation of STAT3 level in cachectic lung cancer patients could be expected. It is known that IL-6 induces STAT3 activation via gp130 receptor dimerization, which recruits STAT3. STAT3 is then phosphorylated, initiating the activation of multiple-genes associated with inflammation (14, 15). However, STAT3 expression is also regulated by different cytokines such as: TNFα, IL-11, IL-22, IFNα and IFNβ and signaling pathways (NF-κβ) (50–52). Our findings indicated that the expression level of STAT3 was significantly higher in cachectic lung cancer patient than non-cachectic patients (p < 0.01) and thus STAT3 could be a therapeutic marker in lung cancer patients. However, the changes in STAT3 expression levels could possibly be regulated by different cytokines and signaling pathways. Thus, the underlying molecular signaling pathways of STAT3 activation and associated cytokines should be further investigated.

Although cachexia is generally observed in pancreatic, gastric, colorectal, lung, and head and neck cancer, recent studies have provided a compelling evidence that there is a relationship between advance metastatic breast cancer and cachexia due to a decrease in muscle mass and excessive weight loss (53–55). Recent studies have also demonstrated that a high level of IL-6 is associated with cachexia, angiogenesis, atrophy, increased catabolism of muscle protein

and resistance to treatment in advanced breast cancer (2, 35, 56). Furthermore, breast cancer patients with high levels of IL-6 compared to the healthy control groups indicate cachexia symptoms and are characterized by weight loss (33, 57, 58). Additionally, the activation of JAK/STAT, PI3K, and MAPK pathways linked to cancer metastasis, tumor cell proliferation, differentiation, apoptosis, angiogenesis and invasion have been identified in patients with breast cancer (59–62). In our study, the expression levels of IL-6 and STAT-3 were significantly higher in cachectic and non-cachectic breast cancer patients than that of the control group, and especially these mRNA levels were up-regulated in breast cancer with cachexia. Thus, IL-6-mediated STAT3 signaling could be a promising biomarker in cachectic breast cancer patients as well as gastric cancer patients.

Conclusion

Our results suggest that STAT3 could be used as a prognostic biomarker in gastric, lung and breast cancer patients with cachexia and IL-6 mediated STAT3 activation in cachectic gastric and breast cancer patients. Additionally, the association between IL-6 and STAT3 levels and cancer types in terms of cancer cachexia was statistically significant.

However, further studies are required to explore IL6-mediated STAT3 activation in larger cohorts of gastric, lung and breast patients with cachexia or other cancer types.

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