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Original Article

The Comparison of Exosome and Exosomal Cytokines between Young and Old Individuals with or without Gastric Cancer

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SUMMARY

Background: Exosomes are small extracellular vesicles secreted by various types of cells. Exosomes play an important role in intercellular communication by serving as vehicles for transferring proteins, lipids, mRNAs, microRNAs, and DNAs to recipient cells. This study investigated the role of exosomes in gastric cancer and the aging process by using a large number of human serum samples from young and aged individuals with or without gastric cancer.

Methods: The age- and cancer-associated changes in exosome levels and exosomal cytokine content were measured. Exosomes in human serum were isolated by using a total exosome isolation reagent, followed by a quantification of isolated exosomes using a MicroBCA assay. Exosomal cytokines were measured by ELISA.

Results: Serum exosome levels are increased by cancer and aging. Exosomal levels of TNF-α and TGF-β are increased, whereas IL-10 levels are reduced in gastric cancer. In addition, the cancer-associated changes in exosomal cytokines remain constant with age, even though aging affects every type of immune cells and immunomodulating factors.

Conclusion: Gastric cancer and aging affect serum exosome and exosomal cytokines levels. Future studies would require the analysis of more diverse cytokines in exosomes derived from multiple types of cancer.

1. Introduction

Exosomes are small extracellular vesicles of 30–100 nm in diameter that are secreted by various types of cells. They can be detected in body fluids such as urine, serum, saliva, and breast milk as well as cell culture media. Initially, exosomes were thought to function in waste disposal, to eliminate unnecessary cellular components. However, studies have revealed that they play an important role in intercellular communication by serving as vehicles for transferring proteins, lipids, mRNAs, microRNAs, and DNAs to recipient cells. Because exosomes are of endocytic origin, they commonly contain endosome-associated proteins including Rab GTPase, SNARE, annexin, and tetraspanins such as CD63, CD81, CD82, CD53, and CD37. In addition, the content of exosomes varies with cell origins and the physiological and pathological conditions of exosome-secreting cells. For example, exosomes released by platelets contain a tissue factor involved in coagulation,[1] whereas those from antigen presenting cells contain major histocompatibility complex class II[2]. Tumor-derived exosomes harbor various cellular factors promoting tumor-growth, angiogenesis, invasion, metastasis, immunosuppression, or drug resistance.[3,4] Because exosomes are highly associated with disease initiation and progression, understanding the diverse exosomal contents and their specialized functions can provide new diagnostic and therapeutic tools for disease control.

During aging, the human body undergoes changes in appearance and declines in physiological function, increasing the chances of developing numerous diseases.[5,6] Aging is largely driven by changes in protein production and turnover, and affects individual body systems to different degrees in an organ-specific manner.[7,8]

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Exosomes have also been found to play a role in cellular senescence.[9] Some studies have reported that senescent cells produce high levels of exosomes, and these senescence-associated exosomes contain immunoregulatory factors and microRNAs that contribute to influence aging process.[10,11] Aging is an important risk factor for cancer, and cancer incidence increases with age. Studies have shown that exosomes are involved in cancer development,[12] progression,[13] severity, and response to treatment by transferring proteins and nucleic acids, including microRNAs,[14] to recipient cells in the tumor microenvironment or in distant regions.[15] However, it has not been studied whether exosomes affect cancer differently in young and aged individuals.

Therefore, in the current study, we examined the age- and cancer-associated changes in exosome and exosomal cytokine content using a large number of human serum samples from healthy population and gastric cancer patients, both including young and old subgroup, resulting in the analysis of 4 subgroups.

2. Materials and methods

2.1. Human serum samples

Human serum samples were kindly provided by Prof. Sun Young Rha (Yonsei University College of Medicine, Seoul, Korea). The serum samples were obtained from 69 young (median age 26 years, range 23–45) and 80 old (median age 75 years, range 63–90) healthy individuals, and 66 young (median age 39 years, range 26–45) and 70 old (median age 72 years, range 65–86) patients with gastric cancer of stages I through IV (Table 1). The patient samples were taken from patients who were diagnosed with gastric cancer and underwent cancer treatment at Yonsei Cancer Center, Severance Hospital (Yonsei University Health System, Seoul, Korea). The normal serum samples were obtained from healthy individuals who visited Yonsei Severanc Hospital for regular health exams and were free from cancer and chronic disease including diabetes mellitus, cystic fibrosis, asthma, immunological disorder, and COPD. Serum samples were prepared by centrifuging tubes with whole blood at 2000 g for 10 min at 4 °C to remove clot after coagulation. The supernatants were carefully removed and stored at −70 °C until analyzed. This study was approved by the Ethics Committee in Yonsei Cancer Center, Yonsei University College of Medicine.

Overall survival (OS) was calculated from the date of gastrectomy to the date of death, while recurrence-free survival (RFS) was defined as the interval between the date of gastrectomy and the date of either recurrence or death.

2.2. Isolation and quantification of exosomes

Exosomes in human serum were isolated by using a total exosome isolation reagent (Invitrogen, Carlsbad, CA, USA), following manufacturer's instructions. Briefly, human serum was treated with 1/5 of isolation agent and incubated at −20 °C over 15 h. After incubation, the mixture was centrifuged at 10000 × *g* for 30 min, and the supernatant was removed. To obtain exosomes with high purity, the pellet was suspended twice in sterilized PBS. For quantification of isolated exosomes, a MicroBCA assay (Thermo Fischer Scientific, Waltham, MA, USA) was performed according to the manufacturer's instructions. The absorbance was then measured at 540 nm using a ThermoMax plate Reader (Molecular Devices, Sunnyvale, CA, USA).

Table 1
Demographic data of young/old gastric cancer patients.

Variables		Total		YOUNG		OLD	
		Total N	N = 136 %	Total N	N = 66 %	Total N	N = 70 %
Age	Median±S.D. (min-max)	65 ± 17.5 (25-86)		39 ± 4.9 (25-45)		72 ± 5 (65-86)	
Sex	Male	85	62.5	33	50.0	52	74.3
	Female	51	37.5	33	50.0	18	25.7
Operation	Yes	102	75.0	48	72.7	54	77.1
	No	34	25.0	18	27.3	16	22.9
Stage	I	42	30.9	22	33.3	20	28.6
	II	17	12.5	7	10.6	10	14.3
	III	37	27.2	17	25.8	20	28.6
	IV	40	29.4	20	30.3	20	28.6
Histology	AWD	15	11.0	2	3.0	13	18.6
	AMD	31	22.8	5	7.6	26	37.1
	APD	62	45.6	35	53.0	27	38.6
	SRC	24	17.6	23	34.8	1	1.4
	Others	4	2.9	1	1.5	3	4.3
Lauren classification	Intestinal	40	29.4	9	13.6	31	44.3
	Diffuse	40	29.4	28	42.4	12	17.1
	Mixed	5	3.7	2	3.0	3	4.3
	Unknown	51	37.5	27	40.9	24	34.3
HER2	Positive	11	8.1	3	4.5	8	11.4
	Negative	70	51.5	34	51.5	36	51.4
	Unknown	55	40.4	29	43.9	26	37.1
Recurrence	Yes	25	18.4	12	18.2	13	18.6
	No	111	81.6	54	81.8	57	81.4
Survival	Death	60	44.1	28	42.4	32	45.7
	Alive	76	55.9	38	57.6	38	54.3

AWD, well differentiated adenocarcinoma; AMD, moderate differentiated adenocarcinoma; APD, poorly differentiated adenocarcinoma; SRC, signet ring cell carcinoma. Several pathologic factors, including tumor histology, tumor type by Lauren classification, and pathologic TNM staging according to the 7th American Joint Committee on Cancer criteria were obtained from the slide review by two individual pathologists at Yonsei Cancer Center. Tumor histology was classified based on Japanese gastric cancer treatment guidelines 2010. HER2 positivity was defined using the HER2 scoring criteria for gastric cancers by Hofmann et al. (Histopathology 52(7):797), and was confirmed using immunohistochemical staining (IHC; Hercep Test, Dako, Denmark) or silver **in situ** hybridization (SISH; Ventana Discovery XT system, Ventana/Roche, USA).

2.3. Enzyme-linked immunosorbent assay

The isolated exosomes were lysed by RIPA buffer (Thermo Fischer Scientific), and exosomal cytokines were assessed by sandwich ELISA. 2 μg/mL of purified mouse anti-human TNF-α antibody (Ab), rat anti-human IL-6 Ab, rat anti-human IL-10 Ab, and rat anti-human TGF-β1 Ab (eBioscience, San Diego, CA, USA) were used for coating the plates. After washing and blocking, the samples were added. The plates were subsequently incubated with biotinylated mouse anti-human TNF-α Ab, rat anti-human IL-6 Ab, rat anti-human IL-10 Ab, and rat anti-human TGF-β1 Ab (eBioscience), respectively, and streptavidin-alkaline phosphatase (BD Pharmigen, San Jose, CA, USA) was added to the plates. Finally, a phosphatase substrate, p-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, MO) was added, and the absorbance of each well was determined by using a microplate reader at 405 nm. Human TNF-α, IL-10, TGF-β1 recombinant protein (eBioscience), and IL-6 recombinant protein (Peprotech, Rocky Hill, NJ, USA) were used as standards.

2.4. Statistics

All experiments were repeated at least 3 times, and data were expressed as median. Significance of the data was determined by Mann-Whitney *U* test using Prism 5.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Age- and cancer-associated changes in exosomes

Human serum samples were classified by donor age, and exosomes were extracted from the serum samples and quantified. 69 young healthy, 66 young cancer, 80 old healthy, and 70 old cancer samples were used for exosome isolation. As shown in Fig. 1A, the number of exosomes per μL of serum was increased in healthy cancer-free individuals during aging.

Consistent with previous studies,[16,17] the number of exosomes was also increased in young patients with gastric cancer (Fig. 1B) compared to young cancer-free individuals, without any correlation between exosome levels and disease severity, as represented by cancer stage (data not shown). However, exosome numbers were not changed in old cancer patients compared to old cancer-free individuals (Fig. 1C).

3.2. Cancer-associated changes in pro-inflammatory cytokines within exosomes during aging

We then quantified and compared the amount of cytokines present in exosomes between young cancer-free individuals and young cancer patients, and old cancer-free individuals and old cancer patients (Tables 2 and 3). We found that the levels of exosomal TNF-α were increased in young cancer patients compared to young individuals without cancer (Fig. 2A). However, there was no difference in exosomal IL-6 levels between young cancer-free individuals and young cancer patients (Fig. 2B). Similarly, the levels of exosomal TNF-α were elevated in old cancer patients compared to the cancer-free elderly, whereas IL-6 levels were not changed (Fig. 3A and B).

3.3. Cancer-associated decrease in exosomal IL-10 in gastric cancer

IL-10 is a cytokine that possesses immunosuppressive and anti-inflammatory properties, but also shows immunostimulatory activity. As shown in Fig. 2C, exosomal IL-10 concentration was decreased in young cancer patients compared to young cancer-free individuals.

Additionally, the cancer-associated decrease in IL-10 was also observed in old cancer patients compared to the cancer-free elderly (Fig. 3C).

3.4. Cancer-associated increase in exosomal TGF-β in gastric cancer

TGF-β is a multifunctional cytokine that can exert tumor-stimulatory or tumor-suppressive effects in a cell type- and context-dependent manner. Our data demonstrated that exosomal TGF-β is increased in young patients with gastric cancer (Fig. 2D). Elevated levels of TGF-β were also found in exosomes from old cancer patients (Fig. 3D).

4. Discussion

In this study, we examined the age- and cancer-associated changes in exosome and exosomal cytokines such as IL-6, TNF-α, IL-10, and TGF-β.

Aging and cancer are long and multidimensional processes of pathophysiology, and they are intertwined in a complex relationship. Molecular pathways of aging and cancers are both associated with an accumulation of DNA mutation, genomic instability, telomere shortening, oxidative stress, apoptosis, etc., and a physiological decline with age results in an increased risk of cancer initiation and progression.[18,19] Consistent with previous reports,[16,17] we observed that the number of exosomes was increased in the serum of young patients with gastric cancer compared to young cancer-free individuals. In addition, the exosome was also increased in aged cancer-free individuals, suggesting that aging and cancer might affect the cellular secretion of exosome in a similar way. Studies have shown that cells under stress condition secrete enhanced level of extracellular vesicles including exosomes.[20]

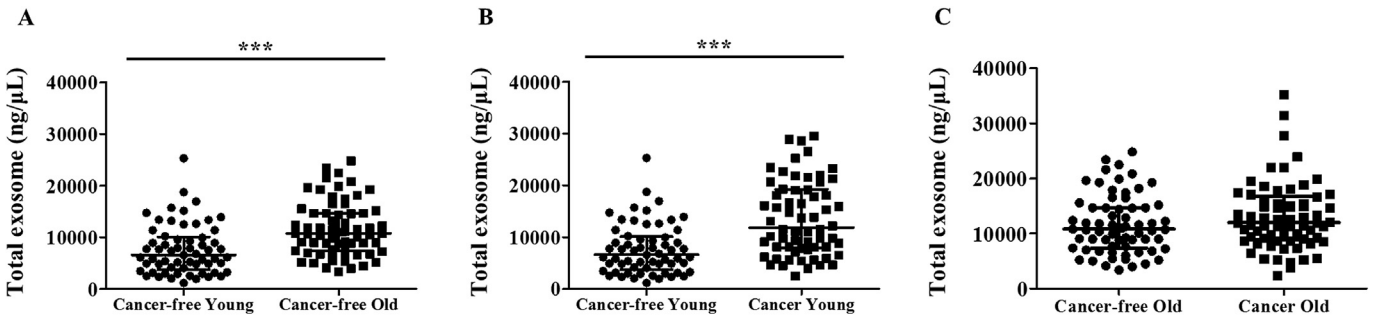

Fig. 1. The comparison of total levels of exosomes between young and aged individuals with or without gastric cancer. Human serum samples were classified by donor's age, and exosomes isolated from the human serum were quantified by MicroBCA assay to compare the levels of exosomes in 1 μL of serum between cancer-free young and old individuals (A), young cancer-free individuals and young cancer patients (B), and old cancer-free individuals and old cancer patients (C). All experiments were performed three times. The bars represent the median value of total levels of exosome in each group with the 25th (lower quartile) and 75th (upper quartile) percentile. ***, $p < 0.001$.

Table 2
The comparison of cytokine expression between cancer-free young individuals and young patients with gastric cancer.

Sample		Median	p-value
TNF-α	Normal Young	0.0300	<0.0001
	Cancer Young	0.2820	
IL-6	Normal Young	2.1240	0.8474
	Cancer Young	1.8510	
IL-10	Normal Young	0.4548	<0.0001
	Cancer Young	0.0780	
TGF-β	Normal Young	1.0960	<0.0001
	Cancer Young	11.570	

Table 3
The comparison of cytokine expression between cancer-free old individuals and old patients with gastric cancer.

Sample		Median	p-value
TNF-α	Normal Old	0.0900	<0.0001
	Cancer Old	0.1940	
IL-6	Normal Old	0.3000	0.0869
	Cancer Old	1.8510	
IL-10	Normal Old	0.1460	<0.0001
	Cancer Old	0.0550	
TGF-β	Normal Old	0.3370	<0.0001
	Cancer Old	5.0760	

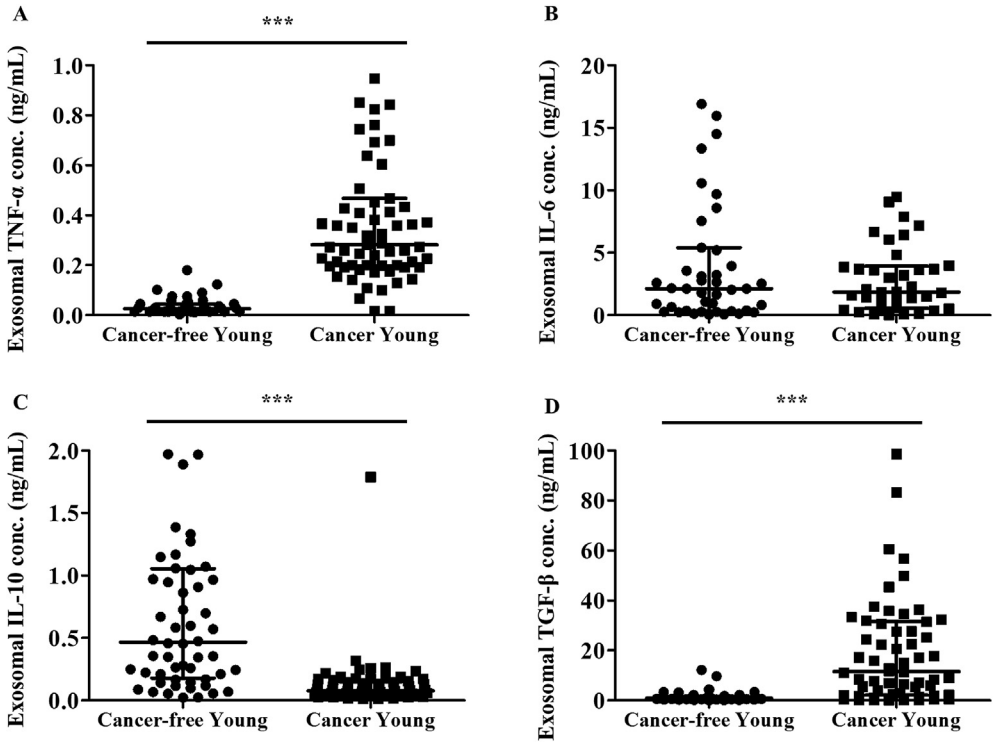

Fig. 2. The comparison of exosomal cytokines between young cancer-free individuals and young cancer patients. Pro-inflammatory cytokines, TNF-α (A), IL-6 (B), IL-10 (C), and TGF-β (D) present within exosomes were measured by ELISA to compare the exosomal levels between young cancer-free individuals and young cancer patients. All experiments were performed three times. The bars represent the 25th (lower quartile), median (middle), and 75th (upper quartile) percentile of each cytokine. ***, $p < 0.001$.

Changes in intracellular calcium level and synaptic activity, p53 activation resulting from DNA damage, and oxidative stress are contributing factors for inducing exosome secretion, and such factors are also related to aging and cancer.[21–24] Conversely, exosome influence aging process and cancer development and progression by transferring various signaling molecules, constituting close interplay between exosome, aging, and cancer.

IL-6 and TNF-α are pro-inflammatory cytokines that are important for cellular signaling and inflammatory reactions. As chronic inflammation promotes tumor development and progression, IL-6 and TNF-α bridge inflammation and tumorigenesis, and promote cell survival, proliferation, invasion, and metastasis. IL-6 and TNF-α are often found at high concentrations in the tumor microenvironment, and their serum levels are elevated in many types of cancer. Soderberg et al. have reported that exosomal TNF-α from melanoma cells induces high levels of reactive oxygen species in T cells, affects the TCR-CD3 complex, and disrupts the activation of CD4 and CD8 T cells.[25] Our results suggest that the increase in

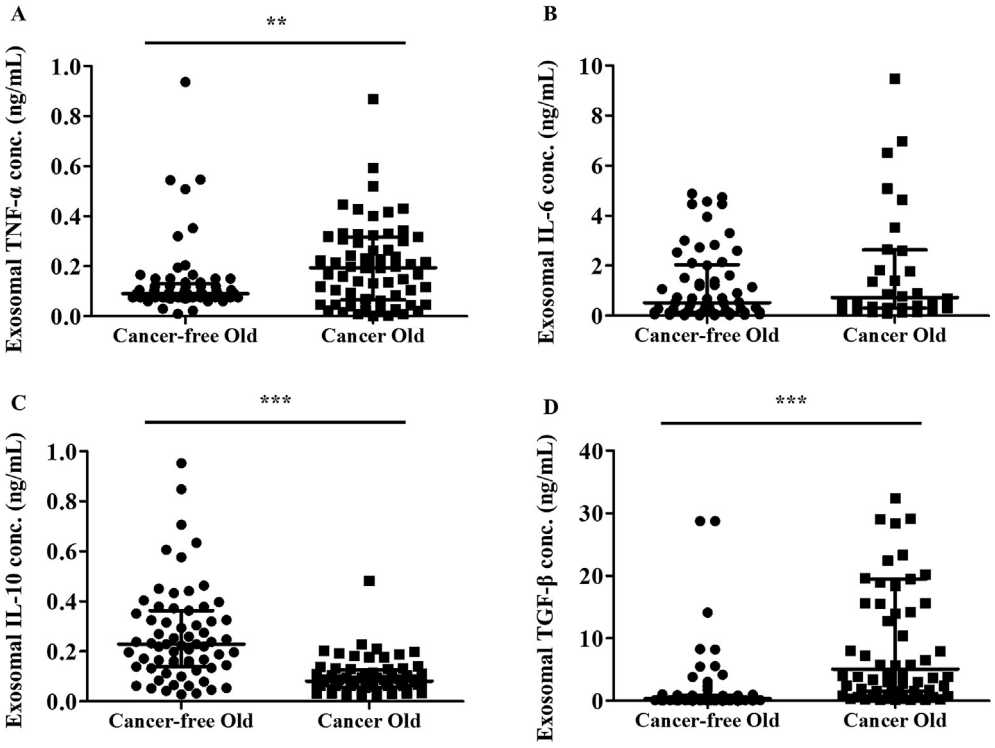

Fig. 3. The comparison of exosomal cytokines between old cancer-free individuals and old cancer patients. Pro-inflammatory cytokines, TNF-α (A), IL-6 (B), IL-10 (C), and TGF-β (D) present within exosomes were measured by ELISA to compare the exosomal levels between old cancer-free individuals and old cancer patients. All experiments were performed three times. All bars here represent the median values, with the lower, middle, and upper quartiles shown). **, $p < 0.01$; ***, $p < 0.001$.

TNF-α concentration within exosomes would help tumor cells escape T cell-mediated antitumor immunity. In addition, although serum IL-6 levels are increased in gastric cancer and even reflects disease status, survival rate, and prognosis, exosomal IL-6 remains unchanged in gastric cancer patients irrespective of age. The data suggest that the changes in serum cytokine levels do not correlate with the levels of exosomal cytokines.

IL-10 is well known for its ability to downregulate the expression of proinflammatory cytokines such as IL-12 and TNF-α, and for its immunosuppressive effects favoring tumor escape from immune surveillance. On the other hand, IL-10 also exerts stimulatory effects on thymocytes, B cells, and mast cells. Owing to its opposing effects, the role of IL-10 in cancer remains controversial. Multiple studies have reported a positive correlation between IL-10 levels and tumor growth and progression in various cancer types, including melanoma, lung cancer, and T/NK-cell lymphomas.[26] Conversely, IL-10 has also been shown to inhibit tumor growth and metastasis in gastric adenocarcinoma, prostatic cancer, and cervical cancer. Nonetheless, many studies have reported high expression levels of serum IL-10 in most types of cancer, supporting the immunosuppressive and anti-inflammatory roles of IL-10[27]. However, serum IL-10 remains constant in healthy individuals during aging.[28] Inconsistent with our results, Wang et al. have reported that exosomal IL-10 levels were increased in small cell lung cancer and non-small cell lung cancer, where it played an essential role in the migration of cancer cells together with TGF-β.[29] The discrepancy in the levels of IL-10 between their and our studies might be attributed to the difference in cancer types. Given the indecisive role of IL-10 in diverse cancers, further analysis of exosomal IL-10 in various types of cancer might yield more conclusive results.

TGF-β plays an important role in immune responses, mainly by suppressing immune responses to maintain self-tolerance and prevent autoimmune diseases. As a pleiotropic cytokine, TGF-β regulates every type of immune cell that constitute the innate and adaptive immunity. TGF-β suppresses the proliferation, differentiation, and effector functions of multiple immune cells, and induces the generation of immunosuppressive cells such as regulatory T cells. Specifically, tumor-derived TGF-β exerts immunosuppressive effects to promote tumor growth, and stimulates angiogenesis.[30] Because of the importance of TGF-β in tumor immunity, the role of exosomal TGF-β has been extensively studied in different types of cancer. The levels of exosomal TGF-β1 have been shown to correlate with the response to chemotherapy in acute myeloid leukemia (AML), and highly metastatic tumor cells secrete higher levels of exosomal TGF-β compared with non-/low-metastatic tumor cell lines. In addition, exosomes secreted from breast cancer cells promoted the accumulation of myeloid-derived suppressor cells through prostaglandin E_2 and TGF-β-mediated pathways. Our data demonstrated that exosomal TGF-β is increased in young patients with gastric cancer, supporting a previous report that TGF-β is enriched in exosomes derived from chronic myeloid leukemia.[31] Considering the supportive role of TGF-β in tumor growth and escape from immune surveillance, our finding of an increase in TGF-β load within cancer-derived exosomes would potentiate tumor progression, metastasis, and immune evasion.

Recently, literature has reported that exosomes and some exosomal miRNAs and proteins are increased in serum of cancer patients such as colorectal, prostate, pancreatic and breast cancers.[32–36] Accordingly, exosomes and exosomal contents are emerging as a new type of biomarkers for cancer diagnosis and treatment, and even some exosome-based cancer diagnostic kits became commercially available for detecting prostate and lung cancers.[37]

Given the changes in the amount of exosome and possibly exosomal contents during aging as observed in this study, such age-associated variations should be considered when applying exosomes to cancer diagnosis and treatment in elder patients clinically.

Taken together, our results demonstrate that serum exosome levels are elevated by cancer and aging in gastric cancer. Exosomal levels of TNF-α and TGF-β are increased, whereas IL-10 levels are reduced in gastric cancer. In addition, the cancer-associated changes in exosomal cytokines remain constant with age, although aging affects every type of immune cell and immunomodulating factor. Nonetheless, this study has some limitation in that it does not provide enough demographic data of healthy individuals except for sex and age due to the administrative issue during sample collection of healthy volunteers. Additionally, comprehensive analysis of exosomal cytokines could not be conducted. Future studies would require the collection of sufficient personal data from healthy individuals and analysis of more diverse cytokines in exosomes derived from multiple types of cancer.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ijge.2018.03.013.

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