Dietary intake of probiotic kimchi ameliorated IL-6-driven cancer cachexia

Jeong Min An,1 Eun A Kang,1 Young-Min Han,1 Ji Young Oh,2 Dong Yoon Lee,2 Seung Hye Choi,2 Duk Hwan Kim3 and Ki Baik Hahm1,3,*

1CHA Cancer Prevention Research Center, CHA Bio Complex, 335 Pangyo-ro, Bundang-gu, Seongnam, Korea
2CJ Food, Gwanggyo-ro, Yeongtong-gu, Suwon 16495, Korea
3Digestive Disease Center, CHA University Bundang Medical Center, 59 Yatap-ro, Bundang-gu, Seongnam 13496, Korea

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Cancer cachexia is a syndrome accompanying weight loss, skeletal muscle atrophy, and loss of adipose tissue in patients with advanced cancer. Since interleukin-6 (IL-6) is one of core mediators causing cancer cachexia and kimchi can modulate IL-6 response, we hypothesized dietary intake of kimchi can ameliorate cancer cachexia. In this study, we studied preemptive administration of kimchi on mouse colon carcinoma cells colon (C26) adenocarcinoma-induced cancer cachexia and explored anti-cachexic mechanisms of kimchi focused on the changes of muscle atrophy, cachexic inflammation, and catabolic catastrophe. As results, dietary intake of kimchi significantly attenuated the development of cancer cachexia, presented with lesser weight loss, higher muscle preservation as well as higher survival from cancer cachexia in mice. Starting from significant inhibition of IL-6 and its signaling, kimchi afforded significant inhibition of muscle specific ubiquitin-proteasome system including inhibition of atrogin-1 and muscle ring finger protein-1 (MuRF-1) with other muscle related genes including mitofusin-2 (Mfn-2) and PGC-1α. Significant inhibition of lipolysis gene such as adipose triglyceride lipase (ATGL) and hormone-sensitive ligase (HSL) accompanied with significant induction of fatty acid synthase (FAS) and sterol response element binding protein 1 (SREBP1) was achieved with kimchi. As gene regulation, IL-6 and their receptor as well as Janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3) were significantly attenuated with kimchi. In conclusion, dietary intake of cancer preventive kimchi can be an anticipating option to ameliorate cancer cachexia via suppressive action of IL-6 accompanied with decreased muscle atrophy and lipolysis.

Key Words: cancer cachexia, C26 adenocarcinoma, kimchi, IL-6, muscle atrophy

Sarcopenia and cachexia are muscle wasting syndromes associated with aging, many chronic diseases, and cancer presenting with weight loss, muscle atrophy, fatigue, and frailty.11–13 Especially, cancer cachexia is characterized by a significant reduction in body weight resulting predominantly from loss of adipose tissue and skeletal muscle, by which tolerance to cancer treatment is markedly impaired and poor quality of life is resulted.2–4 However, cancer cachexia is underestimated and under-recognized, since cancer treatment as well as complicated cachexia is not easy and recovering from the underlying condition causing cancer is impregnable, troublesome cancer cachexia is one of unmet medical needs.5–7 As a scientific approach for cancer cachexia, many attempts have been made to inhibit cancer cachexia by targeting the inflammatory cytokines tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6), which are the molecular biologic mechanisms associated with the development of cancer cachexia.6–7 IL-6 as one of core cachexic factors can lead to promote muscle atrophy-associated genes such as muscle ring finger protein-1 (MuRF-1) and Atrogin-1, resulting from the induction of ubiquitin proteasome system (UPS) E3 ligase genes that mediate the degradation of myofibrillar proteins by the ubiquitin proteasome pathway8–11 and pivotal in generating multiple catabolic catastrophe and weakness.

During long-term follow up of Helicobacter pylori infection in mice model, significant weight loss is observed with the development of chronic atrophic gastritis. In this model, we have found that the dietary intake of kimchi significantly prevented either development of chronic atrophic gastritis or gastric cancer as well as significant attenuation of chronic inflammation-associated sarcopenia.12 In this condition, we have additionally found that kimchi administration significantly decreased IL-6 as well as rejuvenation of precancerous atrophic gastritis.13 Furthermore, since concern regarding gut dysbiosis and sarcopenia is increasing, kimchi is a representative food containing beneficial bacteria, by which we called kimchi as probiotic kimchi.14–16

In this study, under the hypothesis that dietary intake of probiotic kimchi can ameliorate cancer cachexia, we have administered kimchi preemptively in mouse colon carcinoma cells colon (C26) adenocarcinoma-induced cancer cachexia model and found the dietary administration of kimchi preemptively can be anticipating treatment in patients who are at high risk for cancer cachexia.

Materials and Methods

Kimchi production. Preparation of kimchi was based on the standardized kimchi recipe of CJ Food Research Institute at Suwon. Kimchi is made of brined Korean orange cabbage, red pepper powders, garlic, ginger, anchovy juice, radish, green onion and glutinous rice paste. In addition to these basic ingredients necessary for kimchi, additional supplements such as onion, mustard leaf, pear, sea tangle juice and Leuconostoc mesenteroides CJ LM119, Lactobacillus plantarum CJ LP133 were included in kimchi used in this experiment, so called cancer preventive kimchi (epkimchi).

Kimchi processing used in vivo animal model. All of the kimchi samples were lyophilized and freeze-dried kimchi samples underwent an extraction process with 20 times of methanol by stirring overnight. Finally, the kimchi methanol extracts were concentrated by heat evaporation (Eyela rotary evapor system) and stored at 4°C. Since the usual serving dose of kimchi in Korea is approximately 30–70 g/day upon individual taste. The extracted epkimchi was mixed into diet pellet, changed

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E-mail: hahmkb@cha.ac.kr

To whom correspondence should be addressed.
PCR was performed over 30 cycles of 94°C for 30s, 52°C for 30 s, and 72°C for 30 s. Oligonucleotide primers for IL-6, p-JNK, p-ERK, p-PI3K p85/p55, p-AKT, p-mTOR, p-HSL, p-STAT3, p-JAK2, and p-STAT1 were obtained from Bioneer (Daejeon, Korea). Oligonucleotide primers for Atrogin-1, MuRF-1, Mfn-2 and GAPDH were shown in Table 1. The IL-6 expression was measured using the Enzyme-Linked Immunosorbent Assay (ELISA) kit (eBioscience, San Diego, CA) according to the manufacturer’s instructions, taking an mean of the three determinations. The process of IL-6 levels in the supernatant was measured by ELISA, and the significant weight loss, 25% weight loss around 4 weeks and led to mortality thereafter. Therefore, we decided to keep 3 weeks after xenograft. As seen in Fig. 1B, cpkimchi administration significantly reduced the serum level of IL-6 when typical cancer cachexia phenotype presented with around 15–20% weight loss and muscle atrophy was seen. In this condition, as seen in Fig. 1B, cpkimchi administration significantly resisted to cancer cachexia as presented with significant lesser weight loss (p<0.05). Since one of core cachexic mediators was IL-6, as seen in Fig. 1C, significantly increased expression of IL-6 was noted, both IL-6 mRNA and IL-6 protein expressions were significantly increased in Group 2 (p<0.05). However, their expressions in Group 3 were significantly decreased (p<0.05). As much as IL-6 expression in muscle, sera levels of IL-6 were significantly increased in Group 2, but significantly decreased in Group 3 (p<0.05, Fig. 1D). Also, we measured the gp130 IL-6 receptor, significantly decreased expressions were noted in Group 3 (p<0.05, Fig. 1E). Additional measurement of IL-6 signaling, p-STAT3 and p-JAK2, significant inhibitory conditions were noted in Group 3 treated with cpkimchi (p<0.05, Fig. 1F). Finally, we have confirmed these changes with confocal imaging of IL-6. As seen in Fig. 1G, IL-6 expressions were significantly increased in tumor bearing muscle, while the expression of IL-6 was reverted to normal level with cpkimchi administration.

### Table 1. Primer sequences in this experiment

| Primer | Forward | Reverse |
|--------|---------|---------|
| IL-6   | GGG ACT GAT GCT GTG GAC AA | TAA CGC ACT AGG TTT GCC GA |
| Mfn-2  | GCTCGGAGGCACATGAAAGT ATCACGGTGCTCTTCCCATT |
| Atrogin-1 | CTG AAT AGC ATC CAG ATC AGG AGG | TGG ATA AAG TCT TGA GGG GAA AGT G |
| MuRF-1 | AAA TGC TAT GGA GAA CCT GGA | GTC CTT GGA AGA TGC TTT GTA A |
| GAPDH | AAT GTA TCC GTT GTG GAT CT | TCC ACC ACC CTG TTT GTG TA |
Fig. 1. Dietary intake of kimchi to mitigate C26 adenocarcinoma-induced cancer cachexia. (A) Schematic protocol for experiment Balb/c mice were administered with $1 \times 10^7$ cells/ml on side of abdomen. (B) Daily measurement of body weight according to group. (C–F) IL-6 and its signal according to group. (C) RT-PCR for IL-6 and Western blot for IL-6. (D) ELISA for sera levels of IL-6 according to group. (E) Western blot for gp130. (F) Western blot for p-JAK2 and p-STAT3. (G) Immunofluorescence staining for IL-6 according to group, ×100.
Fig. 2. Leg muscle according to group. (A) Representational photo showing both thigh and leg muscle using imaging analysis, individual muscle mass of leg was measured and averaged according to group. The mean size and volume of resected C26 adenocarcinoma xenograft after 3 weeks. (B) Mean amounts of daily food intake were averaged according to group. No significant difference in amounts of pellet diet was noted. (C) Mean tumor mass according to group. No significant difference in C26 tumorgraft. (D) Histopathology of leg muscle. The pathological scores including muscle bundle size, presence of inflammation, and degree of muscle change were shown according to group. (E) Immunofluorescence staining for IgG, ×100.
mass of thigh and gastronecusa was measured in all mice after dissecting skin as seen in Fig. 3A and there was significant difference in mean muscle mass between Group 2 and 3 (p<0.05). Since there is possibility dietary kimchi can improve appetite or suppress cancer growth, we monitored the amounts of diet and tumor size according to group. As noted in Fig. 2B and C, there was no significant difference in either diet intake or tumor size between Group 2 and 3, suggesting kimchi improved cancer cachexia neither through stimulating appetite nor tumor suppression. On pathological analysis of thigh muscle, the mean bundle size of muscle was significantly decreased in Group 2. When we scored pathology based on muscle bundle size, inflammation, and muscle denaturation, the pathology scores of Group 2 were significantly increased, whereas significantly decreased in Group 3 (p<0.01, Fig. 2D). IgG staining of muscle was done to depict the inflamed and denatured status of muscle. As noted in Fig. 2E, IgG staining was significantly increased in Group 2, while significantly decreased in Group 3, signifying cpkimchi significantly restored cachexic muscle.

Usually significant changes of muscle-related UPS were implicated in muscle atrophy of cancer cachexia. As seen in Fig. 3A, atrogin-1 and MuRF-1 mRNA was significantly increased in Group 2 (p<0.001, Fig. 3A). When checked ubiquitin ligase, as seen in Fig. 3B, UPS was significantly decreased with kimchi. Furthermore, mitochondrial repression was noted in Group 2 (p<0.001, Fig. 3B). Mitofusin changes were prominent, as seen in Fig. 3C, Mfn-2 mRNA was significantly decreased in Group 2, while significantly increased in Group 3 (p<0.001). On the other hand, regarding muscle regeneration, kimchi significantly decreased AMPK activation, whereas PCG-1α was significantly restored (p<0.001, Fig. 3D). All of these results suggested kimchi can preserve muscle status and some stimulation to myogenesis.

**Kimchi significantly rescued from cachexia through inhibiting lipolysis.** Looking at the changes of lipid, usually cancer cachexia is associated with increased lipolysis. As seen in Fig. 4A, either ATGL or HSL expressions were significantly decreased with kimchi administration (p<0.005, Fig. 4A), transcriptional changes in this lipolysis was further documented with significantly increases of fatty acid synthase and SREBP-1 (p<0.005, Fig. 4B). All of these findings were validated with confocal imaging of SREBP1. As seen in Fig. 4C, immunostaining of SREBP1 was significantly decreased in Group 2, while significantly conserved in Group 3, telling cpkimchi significantly prevented cachexia-induced lipolysis as well as stimulating lipogenesis.
Kimchi orchestrated cachexia-relevant transcriptional factors. Cancer cachexia is provoked by multi-mechanisms, among which cachexic inflammatory reactions via NF-κB activation had been known a pivotal mechanisms. As seen in Fig. 5A, NF-κB activation was significantly increased in Group 2, increased phosphorylation of NF-κB 65 and NF-κB p50 as well as significantly increased phospho-KB (p<0.001). Also looking at the changes of MAPKs, ERK1/2, p38, and JNK activations were all noted in Group 2 (p<0.01), and these MAPKs were all significantly inactivated with kimchi treatment (p<0.01, Fig. 5B). On the other hand, survival and growth signals consisted of AKT, mTOR and PI3K were all significantly decreased in Group 2, might be associated with muscle atrophy and general weakness, but these signals were all significantly increased with kimchi administration, restoring atrophied muscle in cancer cachexia (p<0.001, Fig. 5C). PTEN expression was the reverse. When looking at PARP to explain decreased muscle bundle, representational measurement of PARP cleavage, PARP cleavage was significantly attenuated in Group 3 (p<0.001, Fig. 5D).

Discussion

In this study, we have documented the dietary intake of kimchi can be an anticipatory intervention in patients with advanced cancer, especially in cancer patients vulnerable to develop cachexia as preemptive intervention. The basis for the current study was initiated with two background, one was significant inhibiting action of our cpkimchi against IL-6 under chronic Helicobacter pylori infection(12) and the other was from analysis of RNAseq to search what our cpkimchi moderate genes for anti-inflammation or cancer prevention (data not shown). As summarized in Fig. 6, C26 adenocarcinoma led to significant cancer cachexia accompanied with deranged muscle regeneration, high IL-6 and related signaling, cachexic inflammation, and lipolysis. However, preemptive administration of kimchi led to significant rescue from cancer cachexia. IL-6 as candidate mediator of cachexia has been shown that different kinds of cancer cells secrete IL-6 and that circulating levels of IL-6 correlate with weight loss in cancer patients in some(11,19,20) and increasing levels of IL-6 in tumor bearing mice.
correlated with the development of cachexia. However, although antibody against IL-6, but not against TNF-α, significantly suppressed cachexia development in experimental model, a clinical trial of IL-6 antibody in lung cancer patients did not have significant effect on loss of lean body mass, although anorexia, fatigue and anemia were prevented. We speculate blocking one single target might be insufficient to prevent cancer cachexia because of multi-factorial and multi-targeted origin of cancer cachexia. In the current study, though kimchi trial also started from blocking IL-6, they afforded concerted action to ameliorate cachexia. The other feature might be preemptive administration to block cachexia.

"Preemptive administration" usually applied in the field of pain control since the preemptive approach of pain management means more than just preincisional analgesia, including the application of multi-factious synergistic medication before pain development. In this condition, the absence of biomarkers to develop cachexia led to rely on clinical findings at present time. However, there had been several publications suggestive of poor prognosis depending on poor muscle mass, weight loss, and poor performance in cancer patients. Until now, our study might be the only study showing preemptive efficacy to ameliorate cancer cachexia upon literature search.

Since muscle wasting can occur systemically in older people, sarcopenia, associated with either a physiological response to malnutrition or many diseases such as chronic obstructive pulmonary disorder, diabetes mellitus, chronic renal failure, congestive heart failure, sepsis as well as advanced cancer, in this condition, the loss of muscle mass and strength developed first, but vicious circle of the loss of muscle function followed, resulted commonly from imbalance between excess protein breakdown and reduced protein synthesis. Based on this pathophysiology, either nutrient enrichment or optimal exercise might be a fundamental approach to prevent muscle atrophy. Though might be feasible in some patients, but this policy is not practical in patients with advanced cancer. Unmet medical need arises to develop drugs targeted either increasing muscle regeneration or inhibiting atrophy in the last few years. For instances, agents increasing the levels of PGC-1α, transcription factor jun-B (JUNB) or SIRT1 to slow muscle wasting in various catabolic status, myostatin to antagonize myostatin-activin A-GDF11 signaling as an autocrine factor limiting muscle size, myostatin and activin A antagonists are under high expectation, but future use of these agents requires more than demonstration of increased muscle mass necessitating covering multiple mechanisms and ultimate safety. With substantial progress, some such as myostatin and activin A antagonists are under high expectation, but future use of these agents requires more than demonstration of increased muscle mass necessitating covering multiple mechanisms and ultimate safety.

In this back-ground, our study that dietary intake of kimchi seems to be very high anticipation because kimchi covers either the possibility of preemptive intake or the exertion of multi-
factorial, pathophysiological actions. As shown in the current study, kimchi afforded resistance to cachexia-weight loss, muscle atrophy, fat loss, and considerable restoration. Mechanistically, they operated muscle-specific proteosomic degradation, IL-6 and its signaling inhibition, lipolysis, and cachexic inflammation. Though we did not show the detailed association between microbiota contained in kimchi and improvement of sarcopenia, kimchi is traditional food that is fermented from vegetables such as Chinese cabbage, radish, red pepper, garlic, ginger, green onion, and fermented seafoods. Microbes involved in kimchi fermentation are lactic acid bacteria, including *Leuconostoc* species and the *Lactobacillus* species as key players responsible for kimchi fermentation. Concerning recent update about “Gut-Muscle Communication Axis,” kimchi contributed to amelioration of cancer cachexia in addition to acknowledged mechanisms of muscle regeneration, anti-lipolysis.

Lipolysis also is one of prominent events implicated in cancer cachexia. Inadera *et al.* studied to clarify the biologic characteristics of lipid-depleting factor in both 3T3-L1 adipocytes and C26 inoculated mouse muscle model and found that reduced quantity of mature SREBP1 without affecting PPAR-γ and C/EBP-α, resulting in increased lipolysis and reduced lipogenesis. Similar findings were noted in the current study, but dietary kimchi significantly restored cachexia-induced fat loss under same molecular changes. In another study by Bing *et al.* using similar cachexia model of MAC16 tumors, adipose tissues from cachexic mice contained shrunken adipocytes and increased fibrosis, similar findings as shown in muscle tissue, shrunken in size, decreases in muscle bundles, and some fibrotic changes. On genetic analysis, major reductions in mRNA levels of adipogenic transcription factors such as C/EBPα and β, PPAR-γ, FAS, acetyl CoA carboxylase, stearoyl CoA desaturase 1, glycerol-3-phosphate acyl transferase, and SREBP1. In the current study, we also found similar changes as shown in Fig. 4.

All of these beneficial anti-cachexic contributions of cpkimchi were covered with pertinent modulation of signal transduction implicated in preventing catabolic catastrophe. First, encouraging mTOR and AKT signaling to overcome sarcomenia with adalimumab, silver linings on the horizon by Ebner & Haehling, is recommended since long-term use of mTOR inhibitors led to a marked loss of muscle mass. As summarized by several investigators and ours in the current study (Fig. 6), molecular substrates and mechanisms underlying the dysregulation of skeletal muscle synthesis and degradation included proinflammatory cytokines such as TNF-α, IL-1, IL-6 and related signaling transduction systems such as NF-κB, MAPKs, acute phase reactants, and myostatin-activin-SMAD pathways.

Considering the facts that more than 30% of patients suffering chronic illness die due to cachexia and more than 50% of patients with cancer die with cachexia and that still convincing drugs or agents are not still available in clinic highlight our results in the current study. Conclusively, we found preemptive and dietary intake of kimchi in patients with high risk to develop cancer cachexia can be of potential regimen accompanied with significant improvement of each catabolic catastrophe, cachexic inflammation, and muscle atrophy. Well-designed RCT should be followed for unmet medical need in cancer cachexia.
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Conflict of Interest

No potential conflicts of interest were disclosed.