Ecrg4 contributes to the anti-glioma immunosurveillance through type-I interferon signaling

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\textbf{ABSTRACT}

Esophageal cancer-related gene 4 (Ecrg4), a hormone-like peptide, is thought to be a tumor suppressor, however, little is known about the mechanism of how Ecrg4 suppresses tumorigenesis. Here, we show that the ecrg4 null glioma-initiating cell (GIC) line, which was generated from neural stem cells of ecrg4 knockout (KO) mice, effectively formed tumors in the brains of immunocompetent mice, whereas the transplanted ecrg4 wild type-GIC line GIC(+/-) was frequently eliminated. This was caused by host immune system including adaptive T cell responses, since depletion of CD4\textsuperscript{+}, CD8\textsuperscript{+}, or NK cells by specific antibodies in \textit{vivo} recovered tumorigenicity of GIC(+/-). We demonstrate that Ecrg4 fragments, amino acid residues 71–132 and 133–148, which are produced by the proteolytic cleavage, induced the expression of pro-inflammatory cytokines in microglia \textit{in vitro}. Moreover, blockades of type-I interferon (IFN) signaling \textit{in vivo}, either depleting IFN-\alpha/\beta receptor 1 or using \textit{stat1} KO mice, abrogated the Ecrg4-dependent antitumor activity. Together, our findings indicate a major antitumor function of Ecrg4 in enhancing host immunity via type-I IFN signaling, and suggest its potential as a clinical candidate for cancer immunotherapy.

\textbf{Introduction}

Tumors are caused by the accumulation of genetic and epigenetic mutations in genes called tumor suppressor genes and proto-oncogenes that normally function as regulators of cell proliferation. In addition to tumors themselves, their surrounding microenvironment, which is composed of extracellular matrix and several types of cells including immune cells, endothelial cells, and fibroblasts, has a role to either promote or inhibit tumor growth.\footnote{Research has demonstrated that the activation of type-I IFN signaling pathway was indispensable for GIC elimination in vivo. Together, our findings identified Ecrg4 as a novel immunosurveillance activator.} However, it still remains elusive how genetic and epigenetic mutations in tumor cells influence the surrounding cells.

Esophageal cancer-related gene 4 (Ecrg4), also known as chromosome 2 ORF 40 (C2orf40) and Augurin, was originally identified as one of the genes whose expression decreased in human squamous esophageal cell carcinomas, compared with their surrounding non-tumor cells.\footnote{We addressed how Ecrg4 acts as a tumor suppressor \textit{in vivo}. Unexpectedly, we found that Ecrg4 from GICs, rather than tumor-surrounding cells, was essential for its tumor-suppressor function. Ecrg4 directly induced the expression of pro-inflammatory factors, including tumor necrosis factor \textalpha (TNF\textalpha) and type-I interferon (IFN), in microglia, an innate immune cell in the central nervous system. Among the factors, we demonstrate that the activation of type-I IFN signaling pathway was indispensable for GIC elimination \textit{in vivo}. Together, our findings identified Ecrg4 as a novel immunosurveillance activator.} Thereafter, it has been reported that \textit{ecrg4} expression was downregulated in various types of tumors such as colorectal cancer, glioma, prostate cancer, and breast cancer because of hypermethylation of its promoter.\footnote{It was demonstrated that Ecrg4 was first produced as a precursor protein of 148 amino acids, and then was proteolytically processed into several different fragments.\footnote{The cleaved Ecrg4 fragment(s) seems likely to transmit growth retardation signals into the cells in an autocrine/paracrine manner, rather than Ecrg4 directly inhibits cell proliferation machinery in the cells.}

Moreover, several papers have shown the growth inhibitory effect of Ecrg4 when overexpressed in cancer cell lines.\footnote{Using the mouse glioma-initiating cell (GIC) models, we addressed how Ecrg4 acts as a tumor suppressor \textit{in vivo}. Unexpectedly, we found that Ecrg4 from GICs, rather than tumor-surrounding cells, was essential for its tumor-suppressor function. Ecrg4 directly induced the expression of pro-inflammatory factors, including tumor necrosis factor \textalpha (TNF\textalpha) and type-I interferon (IFN), in microglia, an innate immune cell in the central nervous system. Among the factors, we demonstrate that the activation of type-I IFN signaling pathway was indispensable for GIC elimination \textit{in vivo}. Together, our findings identified Ecrg4 as a novel immunosurveillance activator.} Together, these findings suggested Ecrg4 as a tumor suppressor.

Unlike other commonly studied tumor suppressor genes, \textit{ecrg4} encodes a peptide hormone-like protein, and is thought to function on cell surface and/or in extracellular fluid.\footnote{Esophageal cancer-related gene 4 (Ecrg4), also known as chromosomal 2 ORF 40 (C2orf40) and Augurin, was originally identified as one of the genes whose expression decreased in human squamous esophageal cell carcinomas, compared with their surrounding non-tumor cells.\footnote{Therefore, the cleaved Ecrg4 fragment(s) seems likely to transmit growth retardation signals into the cells in an autocrine/paracrine manner, rather than Ecrg4 directly inhibits cell proliferation machinery in the cells.}} It
lines by overexpressing sv40LT and hrasL61 in mouse embryonic neural stem cells (NSCs) from ecr4 heterozygous intercrosses, as shown previously. GIC(+/+) from ecr4+/+ NSCs clearly expressed ecr4, whereas its expression level decreased in GIC(+/-) from ecr4+/+ NSCs and was undetectable in GIC(-/-) from ecr4/-/- ones (Fig. 1A). There was little difference in cell proliferation and colony formation in soft agar between three lines (Figs. 1B and C). In addition, overexpression of ecr4 did not induce cell cycle arrest in any of GIC lines (data not shown). Nonetheless, we found that GIC(-/-) killed nude mice faster than other GIC lines (Fig. 1D), suggesting that Ecrg4 stimulated antitumor activity of immune system. It should be noted that tumors formed in WT mice showed more aggressive phenotypes than those in nude mice (Fig. 1E, Figs. 2B and C), and closely resembled to human glioblastoma (GBM), the most malignant type of glioma, with massive hemorrhage, pleomorphism, multinuclear giant cells, mitosis, and necrosis (Fig. 2C), suggesting the critical role of immune system for tumor malignancy.

Since Ecrg4 secreted from the tumor-surrounding cells is thought to prevent tumorigenesis as a tumor-suppressor, we examined the tumorigenicity of both GIC(+/+) and GIC(-/-) in the ecr4/-/- brains. GIC(+/+) and GIC(-/-) formed tumors in both brains and kill mice in a similar way (Fig. S1), suggesting that Ecrg4 from tumor-surrounding cells does not act as a tumor-suppressor in our brain tumor model.

**Ecrg4-stimulated immune responses**

We checked which leukocytes were infiltrated into tumor-bearing brains 2 weeks after implantation of GICs, a time point that almost all mice were alive, by flow cytometry. Transplantation of GIC(+/+) and GIC(-/-) increased number of all immune cells examined, CD8<sup>+</sup>, CD4<sup>+</sup>, NK, CD11c<sup>+</sup>, and B cells, compared with the injection of medium alone (control). CD11c<sup>+</sup> cells increased slightly by GIC(+/+) transplantation compared with GIC(-/-) one, however, there were no clear differences in cell population between GIC(+/+) and GIC(-/-) injections (Fig. 3A).

To identify which cytotoxic immune cells were involved in the GIC(+/-) elimination, we performed cell depletion experiments using specific monoclonal antibodies (Fig. 3B).
Depletion of either CD8⁺, CD4⁺, or NK cells enabled GIC (+/+ ) tumorigenicity in WT mice and all of the antibody-treated mice died by about 40 d (Fig. 3C). Moreover, a combination of anti-CD8⁺ and CD4⁺ Abs accelerated GIC(+/+ ) tumorigenicity and shortened the survival time of the transplanted mice, as GIC(−/−) did at a similar duration. These results evaluated that Ecrg4 apparently activated antitumor immune responses.

**Ecrg4 induced the expression of pro-inflammatory factors in microglia**

Cytokines and chemokines play a pivotal role in antitumor immunity by recruiting immune cells and activating them or by directly killing cancer cells. Previous works have shown that cleaved C-terminal Ecrg4 fragments, Ecrg4(71–148) and Ecrg4(133–148), induced secretion of plasma adrenocorticotrophin in rats and IL-6 from microglia, a primary innate immune cell in brain, respectively, indicating that the processed Ecrg4 fragments have biological functions. We therefore examined which cytokines and chemokines were induced in microglia by Ecrg4 fragments. Using microarray analyses, we found that Ecrg4(71–132) and Ecrg4(133–148) fragments increased the expression of 924 and 470 genes, respectively, while they decreased 682 and 260 genes. Both fragments commonly upregulated and downregulated 225 and 165 genes, respectively (Fig. S2). Among the commonly upregulated genes, we noticed that the expression of many pro-inflammatory cytokines, including interleukin 1 (il1), il6, il12 p40 subunit and ifna, and chemokines strongly increased in the presence of Ecrg4 fragments (Fig. 4A). We confirmed the increased expression of key pro-inflammatory factors, il6, il12 p40 subunit, and ifna, and a type-I IFN ifnb, which activates critical components of the innate and adaptive immune systems underlying cancer immunosurveillance, in the Ecrg4 fragment-treated microglia (Fig. 4B). We further evaluated the secretion of candidate cytokines and chemokines using the multiplex cytokine assay; both Ecrg4(71–132) and Ecrg4(133–148) fragments induced secretion of IL1β, IL12 p40 subunit, TNFα, IL6, granulocyte colony stimulation factor (GCSF), C–X–C motif chemokine ligand 1 (CXCL1), and C–C motif chemokine ligand 5 (CCL5) (Fig. 4C, Fig. S3). Notably, the induction of pro-inflammatory cytokines and chemokines in microglia by Ecrg4(71–132) was much stronger than that by Ecrg4(133–148), although both Ecrg4 fragments similarly induced the expression of many pro-inflammatory cytokines and chemokines.

**Ecrg4 prevented GIC tumorigenesis through the activation of type-I IFN signaling**

Production of IL12 p40 subunit and TNFα by microglia has been shown to contribute to both innate and adoptive antitumor immunity. We therefore examined their roles in our GIC transplantation model. Using anti-IL12 p40 subunit and TNFα depletion antibodies, we found that neither factors had apparent effects on GIC elimination (Fig. 5A).

Since Ecrg4 fragments enhanced the expression of ifnb mRNA in microglia in vitro (Figs. 4A and B), we then investigated the roles of type-I IFN, one of essential cancer immunosurveillance factors, in the GIC model. Using the depletion antibody for IFN-α/β receptor 1 (IFNAR1) (22), a subunit of the type-I IFN receptor, we found that GIC(+/+) formed tumor in the brain of WT mice and killed them (Fig. 5B), as GIC(−/−) did so. To verify the antitumor function of type-I IFN in GIC(+/+) model, we further used stat1-deficient mice, in which type-I IFN signaling is completely abolished. We confirmed that GIC(+/+) formed tumors in the brain of stat1-deficient mice and killed them as GIC(−/−) did so (Fig. 5C).
These results clearly indicated that type-I IFN signaling in host cells was indispensable for GIC(+/+).

**Discussion**

It is now widely accepted that tumorigenesis is regulated by both cancer cell proliferation and communication between cancer cells and their surrounding cells, such as immune cells. Many factors and mechanisms have been shown to be involved in cell transformation, however, it still remains to elucidate how transformed cells, which are normally excluded or destroyed in vivo, evade immunosurveillance and form tumor. Here, we showed that ecrg4-deficient GICs, GIC(+/−), formed tumors in brains of WT mice whereas ecrg4-expressing GICs, GIC(+/+), were frequently eliminated, although both types of GICs similarly formed tumors in nude mouse brains, suggesting that tumor suppression was exerted more by immunosurveillance than simple growth inhibition. We demonstrated that this tumor suppression was dependent on the Ecrg4-induced activation of type-I IFN signaling pathway, which functions for immunosurveillance and tumor elimination. 23-27 We also revealed that Ecrg4 induced secretion of many pro-inflammatory cytokines, including IFNβ, IL6, and TNFα, and chemokines from primary microglia in culture. To generalize the potential of Ecrg4/type-I IFN pathway axis as a therapeutic target in GBM, we have checked the expression of both ecrg4 and type-I ifns in human GBM and lower grade glioma (LGG) using the Cancer Genome Atlas (TCGA) analysis (http://cancergenome.nih.gov), and found that the expression of Ecrg4 and type-I IFNs, α5, α14, and β1, significantly decreased in GBM compared with LGG (Fig. S4). These data support our findings that the Ecrg4/type-I IFN pathway axis prevents GBM, although it is difficult to investigate their relationship directly in human. In addition, it may need to evaluate our findings using other glioma models. Altogether, we concluded Ecrg4 as an important danger-alerting factor, which activates the immune cells to eradicate tumor.
Materials and methods

Animals

Ecr4 KO mice were established by conventional gene targeting procedures at RIKEN CDB. The detailed characterization of Ecr4 KO mice will be reported elsewhere. Heterozygotes were then backcrossed to C57BL/6 mice for at least eight generations. C57BL/6-background Stat1−/− mice were described previously.14,15 Balb/c nude and C57BL/6 mice were purchased from Charles River Japan and CLEA Japan. All mouse experiments were performed following the protocols approved by the Animal Care and Use Committee of Ehime University and Hokkaido University.

Plasmids and chemicals

pCMS-EGFP-H-Ras16,17 and pBabe-Puro-SV40LT were previously described.15 The DNA fragments of mouse Ecr4 and its deletion mutants (corresponding to amino acids 71–132 and 133–148) were amplified by PCR and subcloned into pFuse-hIgG1-Fc2 (InvivoGen). The primers used for amplification were as follows: For mEcr4(71–132), sense primer 5′-TGGATCCGTGGGGACCAATGGCCGC-3′ and antisense primer 5′-TGGATCCATAGT-TGGATCCACCCGGACCGTACGCG-3′; For mEcr4(133–148) sense primer: 5′-TGAATTCGAGCCGGGAAAAGTCGCAG-3′ and antisense primer: 5′-TGAATTCGAGCCGGGAAAAGTCGCAG-3′. Chemicals and growth factors were purchased from Sigma and PeproTech, respectively, except where otherwise indicated.

Establishment of mouse GIC lines and cell culture

Mouse model of GIC lines were generated as previously described.14,15 Briefly, NSCs were prepared from embryonic day 14.5 mouse telencephalon and expanded in DMEM/F12 (Wako, Japan) supplemented with chemicals, bFGF (10 ng/mL), and EGF keeping alternative phenotype (M2) and support tumor progression. Thus, it is crucial to elucidate the molecular mechanism of how Ecr4 modulates immune system including M1–M2 transition, using our GIC model system that is one of best models to study the communication between tumor cells and immune cells in WT mice.

In summary, we demonstrated new tumor suppressor mechanism of Ecr4, which from cancer cells, stimulated host immune system and eradicated tumor through the activation of type-I IFN signal. We have identified Ecr4(71–132) as a strong inducer of pro-inflammatory cytokines from microglia. In thinking about immunotherapy, the restoration of ecr4 expression in tumor cells would be attractive, because the expression of Ecr4 was repressed by DNA methylation without mutation or deletion of ecr4 in many tumors. On the other side, Ecr4 (71–132) fragment can be used as an antitumor peptide, although it should be examined whether Ecr4-induced pro-inflammatory activation causes side-effects. Modulation of Ecr4 signaling might be also applied to cancer therapy. Further studies about identification of Ecr4 receptor(s) and dissection the downstream event of Ecr4 signaling might help find new targets for cancer immunotherapy.

Recently, Lee and colleagues have demonstrated the antitumor function of Ecr4(133–148) that has not only activated both NF-kB signaling pathway and phagocytosis in microglia, but has also recruited monocytes in the tumor.17 Here, we have found that Ecr4(71–132) induced pro-inflammatory cytokines in microglia much stronger than Ecr4(133–148), suggesting that Ecr4(71–132) may be a primary pro-inflammatory inducer, although there is a possibility that both fragments may collaborate for the full induction of pro-inflammatory factors, or their signaling pathways may cross-talk for the activation.

It should be noted that GIC(−/−) tumors progressed in WT mice were more aggressive and showed greater similarity to human GBM than those in nude mice. This data clearly indicated that immune cells played a critical role in tumor promotion, as shown previously.28,29 We demonstrated here that Ecr4 fragments have induced the expression of pro-inflammatory cytokines and chemokines, many of which are expressed in the classically (M1) activated macrophage/microglia,30,31 suggesting that Ecr4 is a novel M1 polarizing factor. Therefore, in the absence of Ecr4, microglia may
GICs were used than those in nude mice. For histopathology, antibodies were administered intraperitoneally (i.p) in doses of 500 μg.

Soft agar assay

The procedure has been described previously. Briefly, the cells were suspended in 0.3% top agar and layered onto 0.6% bottom agar. After the top agar solidified, culture medium was added and the cells were cultured for 20 d with medium changes every 3 d.

Intracranial cell transplantation and histopathology

Indicated number of cells was suspended in 3 μL of HBSS and injected into brains of 6–8 week-old female mice that had been anesthetized with 10% pentobarbital as described previously. When transplanted into WT mice, 10 times more Gfogfp+ cells were produced and purified.14,15 When transplanted into SV40 LT–/- mice, NK cells, and IFNAR1 depletion.

Brain-infiltrating leukocyte (BL) isolation

The procedure has been described previously. Briefly, brain tissues were mechanically minced, resuspended in 70% Percoll (GE), overlaid with 37% and 30% Percoll, and centrifuged for 20 min at 500 × g.

Depletion antibodies

IFNAR1 (clone MAR1-5A3), TNFα (clone MP6-XT22), and IL-12 (clone C17.8) depletion antibodies were purchased from Bio X Cell and Biolegend. Other depletion antibodies, anti-CD4+ (clone GK1.5), anti-CD8+ (clone 53.6.7), and anti-NK1.1 (clone PK136) were produced and purified from each of hybridoma cell lines. Antibodies were administered intraperitoneally (i.p) in doses of 500 μg for IL-12 and TNFα depletion and 100 μg for CD4+ cells, CD8+ cells, NK cells, and IFNAR1 depletion.

Flow cytometry

For cell-surface molecules, cell samples were stained with fluorescent dye-conjugated mAb against selected markers on ice. Then, cells were harvested and stained with 7-AAD, anti-CD4+, -CD8+, -NK1.1, CD11c, and B220 (BD Bioscience). Data were acquired by FACSCant II and analyzed with FlowJo software.

Generation of Fc fusion protein

HEK293T cells were transfected with the expression construct using polyethylenimine (PEI). Two days after transfection, conditioned medium was collected, centrifuged, passed through a 0.45-μm filter membrane. Fc fusion protein was purified by protein A-Sepharose 4 Fast Flow (Amersham Biosciences). After dialysis against PBS, purity was checked by SDS-PAGE and CBB staining.

RT-PCR

First-stranded cDNA was synthesized using Transcriptor Reverse Transcriptase (Roche). PCR reactions were carried out in a total volume of 10 μL containing 1 μL of first strand cDNA using ExTaq (Takara). RT-PCR was carried out as described.15 Primers used for amplification of specific genes were as follows: For gapdh, sense primer: 5′-ACCACAGTCATGCCATCAC-3′, antisense primer: 5′-TCCACCACCCTGTTGCTGTA-3′. For mouse ecrγ4, sense primer: 5′-ATGAGCAACCTGCTCTGGCGG-3′, antisense primer: 5′-TTAACATGCATAGTTGACACT-3′. For exogenous hras, sense primer: 5′-AGCACGAATAAAGCTTGTGGTG-3′, antisense primer: 5′-ATAAACCCTCATAAAAGGGAAG-3′. For SV40 LT, sense primer: 5′-ATGGATAAATTTTTAACAGAGAG-3′, antisense primer: 5′-TTATGGTTCCAGTTGTCAGGG-3′.

Real-time PCR was performed by using the LightCycler system (Roche) and LightCycler TaqMan Master (Roche) according to the manufacturer’s protocol. Samples were normalized to the housekeeping gene β-actin according to the ΔCt method: ΔCt = ΔCt sample - ΔCt reference. Percentages against the control sample were calculated for each sample. Primers used for amplification of specific genes were as follows: For β-actin, sense primer: 5′-AGCCATGTACGTAGCCATCCA-3′, anti-sense primer: 5′-TCTCCGGAGTCCATCACAAATG-3′, probe: 5′-TGTTCCCTGTATGCCCTGGTGTCACCA-3′. For IFN-β, sense primer: 5′-ATGAGTGTGTTGTCGAGGCG-3′, antisense primer: 5′-TGACCTTTTCAATGCTAGATCCA-3′, probe: 5′-AACATCACGGAGCCACTCTGGGGA-3′. IL12p40, sense primer: 5′-TGAACAGCGTGTTGGAAGC-3′, antisense primer: 5′-GGGGGTCTGTGGTATGGA-3′, probe: Roche Universal probe #74. TNFα, sense primer: 5′-GTTCCTCTTCAGGCGA-3′, antisense primer: 5′-TCCTGTATTAGAATACGCG-3′, probe: 5′-TACGTGCTCTCACCACA-3′. IL6, sense primer: 5′-GAGGATAACCCTCCCAACAGACC-3′, antisense primer: 5′-AAGTGCTACTCTGTTATCAG-3′, probe: 5′-GAGGATGCGCTTGAGAGC-3′.

Cytokine and chemokine determinations

For analysis of microglia produced cytokine and chemokine profile, the supernatants were obtained from primary microglia culture 24 h after stimulation by 20 μg/mL of Ecrγ4 fragments. Cytokine and chemokine concentrations were measured with Bio-Plex Pro Mouse Cytokine 23-plex Assay, according to the supplier’s protocols (Bio-Rad).

Microarray hybridization and data processing

Total RNA was extracted from mouse microglia treated with 20 μg/mL of Ecrγ4 fragments for 3 h using the TRIzol Plus...
RNA Purification System (Invitrogen). Purified RNA was then amplified and labeled with Cyanine 3 using the one-color Agilent Low Input Quick Amp Labeling Kit (Agilent Technologies) following the manufacturer’s instructions. Labeled cRNAs were fragmented and hybridized to the Agilent mouse GE 8 x 60 K Microarray. After washing, microarrays were scanned with an Agilent DNA microarray scanner. Intensity values for each scanned feature were quantified using Agilent feature extraction software, which performed background subtractions.

Normalization was achieved using Agilent GeneSpring GX version 13.1. After normalization, hierarchical sample clustering of the expressed genes was performed with the Euclidean distance and Ward’s linkage methods (Agilent GeneSpring GX). The microarray data have been submitted to NCBI GEO and available under GSE87376.

**TCGA analysis**

Each 27 microarray data of human GBM and LGG were obtained from TCGA and analyzed for the expression levels of ecr4g, ifna5, ifna14, and ifnb1.

**Statistical analyses**

Statistical analyses were carried out using GraphPad Prism or Microsoft Excel software. Data were presented if not indicated elsewhere as mean ± SD.

**Disclosure of potential conflicts of interest**

S.T., K.E., and U.K are employees of ASUBIO pharma Co., Ltd. The other authors declare that no competing interests exist.

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**Author contributions**

T. Moriguchi designed and performed the experiments, analyzed the data, and wrote the manuscript. S.K. and H.K contributed to the immune cell experiments. S.K.M. performed the culture cell experiments. T. Miki was involved in interpreting data. S.T., K.E., and U.K. performed Microarray and BioPlex assays and data interpretation. T.K. performed experimental design, data analysis, and wrote the manuscript.

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