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Signaling adaptor protein Crk is indispensable for malignant feature of glioblastoma cell line KMG4

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Abstract

Signaling adaptor protein Crk has been shown to be involved in pathogenesis of human cancers including brain tumor where Crk was reported to be overexpressed. In this study, we addressed whether Crk is indispensable for malignant phenotype of brain tumor. In 20 surgical specimens of glioma, mRNA of both *CrkI* and *CrkII* was found to be elevated in malignant tumor. To define a precise role of Crk, we have established Crk-knockdown cell lines of glioblastoma KMG4 by siRNA, and early phase of cell adhesion to laminin was found to be suppressed. Wound healing assay revealed the decreased cell motility in Crk knockdown cells, and suppression of both anchorage-dependent and -independent growth were demonstrated in these cells. Furthermore, *in vivo* tumor forming potential was also markedly suppressed. These results suggest that Crk is required for early attachment to laminin, cell motility, and growth of glioblastoma cell line KMG4.

Keywords: Crk; Adaptor; Signaling; Brain; Glioblastoma; Cancer; SH2; Invasion; Adhesion; Motility

Crk belongs to an adaptor family of proteins composed of SH2 and SH3 domains [1]. CrkII consists of an SH2 and two SH3 domains (SH2–nSH3–cSH3), and alternative splicing variant composed of an SH2 and an SH3 domains (SH2–SH3) was referred as CrkI [2]. Crk transmits signals from tyrosine-phosphorylated proteins including the components of focal adhesion, growth factor receptors, and signaling scaffold proteins, by binding to them via SH2 domain [1]. Crk associates with GEFs (guanine-nucleotides exchange factors) such as C3G and Dock180 those activate Rap1/R-Ras and Rac, respectively, and control cell adhesion, proliferation, and motility [3,4]. Crk has also been reported to regulate Fak tyrosine kinase and PI-3 kinase [5,6]. Crk is known to be overexpressed in various human cancers [7], and recently, Crk was shown to play an essential role for malignant potentials of human ovarian cancer cells, synovial sarcoma cells, and breast cancer cells [8–10].

Brain tumor, especially malignant glioma, is one of the most aggressive tumors in human, and in spite of the recent advancement of therapeutic reagents [11], no curative therapy has not yet been established. In WHO classification, gliomas are classified to Grade I to IV, in which Grade I and II are considered as benign, and Grade III tumor and Grade IV as glioblastoma (GB) are malignant [12]. Genetically, *EGFR* amplification, $p16^{INK4A}$ deletion, and *PTEN* mutations are shown to be associated with primary glioblastoma which initially arise as Grade IV tumor, whereas *p53* mutation are known to be found in secondary glioblastoma in which tumor arise initially as benign and recurrence make it being aggressive [13,14]. In glioma cells, Ras/Erk pathway and PI-3 kinase/Akt pathway are known to contribute to cell proliferation and survival [13].

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In addition, characteristic features of malignant glioma are its elevated cell motility and invasion those are currently known to be regulated by various molecules such as extracellular matrices, integrins, matrix metalloproteinases, growth factor receptors, focal adhesion kinases, PI-3 kinase, small GTPases, and transcription factors [15–17].

As Crk is known to localize to focal adhesion, activate PI-3 kinase, and interact with growth factor receptors leading to regulation of cell motility, we examined whether Crk is overexpressed in malignant human gliomas and is essential for their malignant potential.

Materials and methods

Clinical samples and reverse transcriptase (RT)-PCR. Brain tumor specimens were obtained from patients at the Kashiwaba Neurosurgical Hospital (Sapporo, Japan) and the Iwamizawa Municipal General Hospital (Iwamizawa, Japan) under the informed consent and pathological diagnosis was established in our laboratory. Total RNA was isolated with the TRI Reagent (Sigma, St. Louis, MO, USA) and reverse transcribed into cDNA using the oligo-dT primer (Invitrogen, Carlsbad, CA, USA) and the Superscript II (Invitrogen). The levels of *CrkI and CrkII* were analyzed by PCR with the *KOD plus* DNA polymerase (Toyobo, Tokyo, Japan). Primers used in this experiment included: 5′-GCA GTG GTG GAA TGC GGA G-3′, and 5′-CTG TTG AAC TAT ACT CAG CTG AAG T-3′ for human *CrkI* and *CrkII* (The sizes of PCR products were 260 bp for *CrkI* and 429 bp for *CrkII*); 5′-CTC ATG ACC ACA GTC CAT GC-3′ and 5′-TTA CTC CTT GGA GGC CAT GT-3′ for human *glyceraldehydes-3-phosphate dehydrogenase (GAPDH)*.

Cell culture. The glioblastoma cell line, KMG4 was kindly provided by Dr. Kazuo Tabuchi (Saga University, Japan) [18]. U87MG, U251MG, and T98G cells were obtained from the American Type Culture Collection (Manassas, VA, USA). All cells used were maintained in Dulbecco's modified Eagle's medium (Seikagaku Co., Tokyo, Japan), supplemented with 10% fetal bovine serum. For establishment of Crk-knockdown glioma cells, the pSUPER-vector [19] expressing small interference RNA for human Crk [20] was transfected into the KMG4 cells by means of Fugene 6 (Roche, Indianapolis, IN, USA). Cells were cultured in the presence of 500 ng/ml puromycin (Sigma) and drug-resistant clones were isolated.

Assays for cell adhesion and wound healing. Cells were cultured on 96-well plates coated with laminin, collagen, hyaluronic acid, or fibronectin, and adhesion assay was performed as described previously [8]. Cells cultured on cover glasses coated with laminin, collagen, or fibronectin were also subjected to actin cytoskeleton analysis (phalloidin staining) as described previously [9]. The wound healing assay was performed as described previously [21].

Soft-agar colony formation assay and xenograft propagation. Soft-agar colony formation assay and xenograft propagation were carried out as described [22,23]. All animal procedures were performed according to the protocol approved by the institutional Animal Care and Use Committee at Hokkaido University Graduate School of Medicine.

Histological analysis and immunohistochemistry. Formalin-fixed paraffin-embedded tissues, including human glioma specimens and the KMG4-derived xenografts, were sectioned and stained with haematoxylin and eosin (H&E) using standard protocol. Immunohistochemistry was performed using anti-Crk (Transduction Laboratories, Lexington, KY, USA) and anti-Ki67 (MIB1; Dako, Glostrup, Denmark) antibodies.

Immunoprecipitation and immunoblotting. Protein determination, immunoprecipitation, SDS–PAGE and immunoblotting were carried out as described previously [9]. Antibodies were obtained from the following sources: anti-phosphotyrosine (PY20 and RC20H), anti-p130^{Cas}, anti-paxillin, and anti-Crk antibodies (Transduction Laboratories, Lexington, KY, USA); anti-C3G (C19), anti-DOCK180 (H4), and anti-Crk-L (C20) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-actin antibody (Chemicon International, Temecula, CA, USA); anti-Flag (M2) antibody (Sigma).

Results

Increased expression levels of Crk mRNA in malignant gliomas

In an investigation of Crk functions in human glioma, the levels of *CrkI* and *CrkII* mRNA were analyzed by semi-quantitative RT-PCR using 4 benign (Grade I, $n = 1$; Grade II, $n = 3$) and 16 malignant (Grade III, $n = 4$; Grade IV, $n = 12$) gliomas. In benign gliomas, no increase of either *CrkI* or *CrkII* mRNA was observed compared to those in normal brain tissue (Fig. 1A, lanes 2–5). However, in malignant tumors, 5 cases showed an increase of *CrkI* (Fig. 1A, lanes 7, 8, 12, 15, and 18), and 3 cases showed an increase of both *CrkI* and *CrkII* (Fig. 1A, lanes 19–21). The overexpression of Crk in glioblastoma was confirmed by immunostaining (Fig. 1B). In 5 cases of malignant gliomas, *Crk* mRNA was hardly detectable (Fig. 1A, lanes 6, 9, 11, 14, and 16).

Establishment of Crk-knockdown KMG4 glioblastoma cell lines by siRNA

To investigate the roles of Crk in malignant gliomas, we employed glioblastoma-derived cell lines, including U251MG, U87MG, KMG4, and T98G cells, and protein levels of Crk and its related molecules such as Crk-like (CrkL), C3G, Dock180, paxillin, and p130^{Cas} were analyzed. No significant difference in the protein levels of Crk, CrkL, Dock180, and paxillin were detected, but levels of C3G and p130^{Cas} were increased in T98G cell lines (Fig. 2A). The tyrosine-phosphorylation of p130^{Cas} was detected in all cell lines, but the amount of p130^{Cas} which bound to Crk is almost equal in U251, T98G, and KMG4 cells and that is lower in U87 cells (Figs. 2A and B).

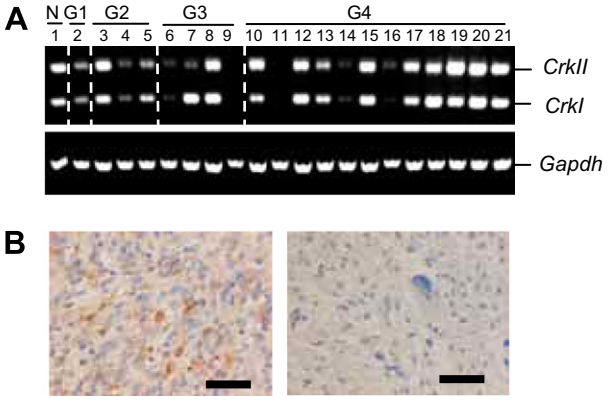

Fig. 1. Expression of Crk in human gliomas. (A) The mRNA levels of *CrkI* and *CrkII* in surgical specimens were analyzed by semi-quantitative RT-PCR. Twenty gliomas including WHO Grade I (G1, $n = 1$), Grade II (G2, $n = 3$), Grade III (G3, $n = 4$), and Grade IV tumors (G4, $n = 12$) were tested. N, normal brain. (B) Immunohistochemical staining was performed with an anti-Crk antibody on archival tissue sections. The representative images of Crk positive (left) and negative tumors (right). Scale bar, 100 μm.