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学 位 論 文 (要 約)

Analysis of mechanism of adaptor protein Crk-induced epithelial-mesenchymal transition (EMT) and its implication to human cancer metastasis

(アダプター分子Crkによる上皮間葉移行機構とヒト癌転移誘導におけるその役割の解明)

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Thesis Summary

Lung cancer is one of the most common cancers globally with an increasing incidence, and remains one of the most dominating causes of malignancy related mortality in the world, significantly because of early aggressive metastasis, as the survival outcome of lung cancer is less than 15% beyond 5 years. Non-small-cell lung cancer (NSCLC) is the major type, accounting for approximately 80% of all lung cancer cases, with lung adenocarcinoma being the most common kind. Bringing a new understanding of the molecular mechanisms and identification of new molecular targets for therapeutic purposes of this lethal disease into an urgent priority. The process of epithelial-to-mesenchymal transition (EMT) and the CT10 regulator of kinase (Crk) adaptor protein family including CrkI and CrkII share several pivotal properties that implicates in cell proliferation, morphogenesis, invasiveness, and metastasis. Crk promotes an epithelial–mesenchymal-like transition in MDCK kidney epithelial cells in the presence of human growth factor (HGF) and epidermal growth factor (EGF), however; the precise mechanisms for inducing mesenchymal features have remained obscure. EMT, the fundamental biological process of tissue and organ formation in embryogenesis, has been reported to be a major component and regulator for the progression of cancer metastasis and morphogenesis machinery and networks in several types of cancer including lung adenocarcinoma. This mechanism is supported by the observation of the mesenchymal phenotypical changes and EMT signature which includes the expression of the main regulatory transcriptional factors Snail and Slug, and the loss of epithelial marker E-Cadherin and the gain of the mesenchymal marker N-Cadherin, what is known as the cadherin switch, beside other mesenchymal markers like Vimentin and Fibronectin which are also increase. TGF-β1 and its SMAD2/3 active signalling is a role player in the induction process of EMT and the resultant EMT related metastasis and tumor microenvironment considered as one of the major TGF-β1 sources contributing in the EMT induction of tumor cells and the increase of tumor invasiveness and metastasis. TGF-β1 and

growth factors like HGF, EGF and PDGFA beside so many others, considered to be the frontline components of the microenvironment that goes in direct contact with tumor cells, this interaction leads to activation of several signaling pathways and cellular transformation like EMT in consequence. These major events are prominent in the invasive front of tumors, from where the tumor begins to metastasize. Although Crk has been reported to induce EMT like phenomenon including cytoskeletal modification or cell spreading, and Crk family of adaptors reported indirectly to be part of the EMT networks, but up to date there is no molecular mechanisms studied if Crk is integrating with the microenvironment components like TGF-β1 to induce or regulate EMT, and the precise role for Crk family of adaptors in the process of metastatic initiation remain to be elucidated.

In this study, immunohistochemical analysis revealed an increase in the expression of Crk especially at the invasive front of NSCLC specimens which was correlated with lower expression of E-cadherin and poor prognosis suggesting that Crk expression could be established under the signaling regulators of the invasive front alongside with EMT and this interaction has a remarkable role in the initiation and ignition of invasiveness process resulting with poor clinical outcome. Immunoblotting analyses of human lung adenocarcinoma and lung squamous cell carcinoma cells showed predominant CrkII protein expression which was similar to the previous studies performed on ovarian, breast cancer and synovial sarcoma, mean-while the expression levels of Crk binding proteins such as; p130Cas, paxillin Gab1, Dock180 and C3G were variable, these results suggesting a possible contribution of Crk-related signaling pathway in NSCLC. In *in vitro* experimental setting mimicking the tumor microenvironment, Crk promoter activity was increased after stimulation with several growth factors and cytokines such as EGF, PDGFA, TGF-β1, and IL-2, corresponding to enhanced CrkI protein expression especially by TGF-β1 indicating that Crk expression is regulated by multiple microenvironmental components as suggested by our clinical observation and revealing a key role of

Crk and TGFβ1 signaling axis in the invasiveness of lung cancer cells in the invasive front. To unveil the distinct functions of Crk family of proteins, CrkI and CrkII were separately overexpressed in A549 lung adenocarcinoma cells using the lentiviral system. Either forced expression of CrkI and CrkII was sufficient to induce mesenchymal features with increased motility and invasion, CrkII showed higher motility and migration ability than CrkI cells, which was further exemplified in immunofluorescence imaging of these cells cytoskeleton, as CrkII overexpressing cells presented with more lamellipodia formations compared to CrkI overexpressing cells. CrkI on the other hand presented with high but not significant abilities of invasion and metastasis *in vitro* when compared with CrkII overexpressing cells, this limited over step explained by the greater production of ECM degrader MMP2. Tumor metastasis in *in vivo* model was remarkably greater in CrkI overexpressing cells than CrkII, corresponding to the integration of CrkI in more efficient way with TGF-β1 signalling as exposure of the tumor cells to TGF-β1 from the platelets and the microenvironment produce more CrkI expression, resulting with higher metastatic ability, also CrkII overexpressed cells made metastasis less than CrkI overexperession cells mostly because of the reversible conversion of CrkII between an activated and an inactivated form by intramolecular binding *via* the phosphorylated Y211. Our results also showed that CrkI promoted phosphorylation of c-MET, Gab1, and $p130^{Cas}$ which also induce EMT-like phenomenon and facilitate metastasis as previously reported. Interestingly, in an experimental setting using inhibitors of Rac1 and RhoA the members of the Rho family of small GTPases, expression of EMT-related molecules was strictly and exclusively regulated *via* Rac1/Snail-dependent regulation of E-cadherin and Fibronectin, RhoA/Slug-dependent induction of N-cadherin, and both pathways collaborated for MMP2 expression. Expression of TGF-β1 and TGF-βRI was also regulated in Rac1- and RhoA-dependent manner and remarkably, CrkI overexpression showed upregulation of p-SMAD3 and p-ERK1/2, while CrkII overexpressing cells showed upregulation of only p-SMAD3. This activation of SMAD, but not ERK,

was abrogated using Rac1 specific inhibitor. Our results also confirmed this integration of Crk and TGF-β1 signaling, as exogenous TGF-β1 synergized Crk induced EMT and antagonizing TGF-β1 signaling using TGF-βR specific inhibitor abolished this effect.

In summary, molecules and signaling cascades responsible for induction and facilitation of metastasis through EMT are important targets for future cancer therapy. Crk adaptor proteins and TGF-β1 signaling are considered as important members in the metastatic process, although their integration or cross-talk signaling remained unclear, urging a decisive analysis of these two prominent molecules. Here we show that CrkI and CrkII are sufficient to induce EMT and initiate metastasis by two parallel pathways synergizing each other, one is EMT induction via Crk/Rac1/Snail and Crk/RhoA/Slug, the other is induction of TGF-β1 signaling and the formation of a feedback activation loop between Crk and TGF-β1, especially CrkI. Nevertheless, CrkI also showed that it is sufficient to activate ERK pathway and phophorylate Met and Gab1, implying that CrkI is more in favor of aggressive forms of cancer. We also demonstrated that Rac1, ROCK and TGF-βR inhibitors are all potentially active against the molecular mechanism of metastasis, showing the importance of these inhibitors in future adjuvant therapy of lung adenocarcinoma. These novel finding will help to concentrate on the importance of Crk as a target for cancer therapeutics especially after the indicated correlation of Crk expression in the invasive front and poor prognosis.