主論文の要旨

CEBPγ facilitates lamellipodia formation and cancer cell migration through CERS6 upregulation

（CEBPγ は CERS6 遺伝子の発現上昇を通じて
ラメリポディア形成とがん細胞遊走を促進する）

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【Introduction】

Lung cancer is the most common cause of cancer death worldwide, with an estimated 1.6 million deaths each year. The initial step of metastasis is dependent on factors related to migration and invasion. Ceramide synthase 6 (CERS6) expression has effects on cellular ceramide constitution to upregulate the C16 ceramide level, resulting in stimulation of cell migration and invasion activities through RAC1-positive lamellipodia formation.

To date, CEBPγ has been reported to be a regulator of cellular stress response networks and senescence, and an inflammatory suppressor, as well as a transcription factor that induces myeloid differentiation arrest in acute myeloid leukemia cases. CEBPγ-mediated regulation mechanism in lung cancer development remains poorly investigated.

【Methods】

The BEAS-2B, an immortalized human lung epithelial cell line as well as NCI-H460-LNM35 (LNM35), PC3, HEK293 and LNCaP cell were used in this study. ChIP assay was carried out to analyze binding pattern between CEBPγ or YBX1 and Y-box according to manufacturer's instructions. Protein levels were measured by western blotting. Immunofluorescence and Mass spectrometric analysis were performed to evaluate ceramide amount.

【Results】

We screened for cis-acting elements in the promoter region using prostate cancer LNCaP cells, which were associated with elevated CERS6 expression (Figure 1A). Analysis to determine transcription start sites (TSSs) showed that those located at 157 and 221 bases upstream from the first exon. In analyses following those results, luciferase constructs using the -1728 bp, which contains the CERS6 promoter region between -1728 and +157 (Figure 1B), to -162 bp regions showed similar promoter activities, whereas those using the -122 bp and -112 bp regions were associated with only a moderate increase and those using the -88 bp to+1 bp regions were associated with a significant decrease (Figure 1C), suggesting the presence of positive element(s) between the -112 and -88 regions. We introduced base substitutions in each of the two putative cis-elements, *i.e.*, Y- and GC-box (Figure 1B), and performed luciferase analysis. In both LNCaP and the LNM35 lung cancer cell lines, disruption of the Y-box sequence resulted in significantly reduced promoter activities, while disruption of the GC-box did not have such an effect (Figure 1D, E). Parallel analysis using a clinical data set was used to screen for transcriptional factors with gene expression profiles correlated with CERS6. Among the genes that showed significant correlations, five coded Y-box binding proteins were noted (Figure 1F, Table 1).

To investigate whether CERS6 expression is controlled by any of those gene products, each was knocked down and CERS6 expression was examined (Figure 2A-D). Following separate siRNA treatments with CEBPγ and YBX1, the CERS6 expression level became decreased in LNM35 cells, while the effect of CEBPζ, NFYA, or NFYB suppression seemed to be only

marginal or increased. Furthermore, the suppressive effect of the Y-box mutation seemed to be neutralized in siCEBPγ- or siYBX1- treated cells (Figure 2E-G), suggesting that CEBPγ and YBX1 directly or indirectly interacted with Y-box. ChIP assay results showed that the anti-HA antibody precipitated a greater number of CERS6 promoter regions than the control, indicating specific binding between CEBPγ and Y-box. In addition to this specific activity, CEBPγ may have general DNA binding activity, because irrespective of the primer sets used, the anti-HA antibody precipitated greater numbers of target regions as compared to the IgG controls (Figure 2H, top). On the other hand, our analysis showed only non-specific binding activity for YBX1 (Figure 2H, bottom). Together, these findings suggest that CEBPγ upregulates CERS6 via specific binding to Y-box, while YBX1 may exert Y-box interaction through one or more other factors.

To understand whether CEBPγ and YBX1 have effects on C16 ceramide levels through regulation of CERS6 expression, MS analysis was performed under knockdown conditions. CEBPγ knockdown consistently reduced the amount of C16 ceramide, whereas the effect of YBX1 knockdown was not significant (Figure 3A). In the immunocytochemistry experiment, cells were stained with an anti-ceramide antibody and then ceramide amounts in microstructures were visualized. The results showed that knockdown of either CEBPγ or YBX1 reduced lamellipodia ceramide levels, while the phenotypes were partially rescued by ectopic addition of C16 ceramide (Figure 3B, C). These findings suggest that both CEBPγ and YBX1 have effects on ceramide levels through CERS6 expression in lamellipodia structures. The effects of CEBPγ and YBX1 expression on lamellipodia formation efficiency were quantitated by determining RAC1- and PKCζ-positivity.

In LNM35 cells, siRNA treatment against either one of the transcriptional factors reduced RAC1-positive lamellipodia formation, which was partially rescued by C16 ceramide (Figure 4A-D). Interestingly, under the same rescue conditions, migration activities were partially recovered in a dose-dependent manner when the cells were treated with siCEBPγ and C16 ceramide (Figure 4E), while no such rescue phenotype was observed with the combination of siYBX1 and C16 ceramide (Figure 4F). The involvement of CEBPγ as an upstream regulator of CERS6 was further examined by a biochemical method (Figure 4G). Knockdown of either CEBPγ or CERS6 in LNM35 cells was found to be associated with a decreased amount of active RAC1 protein. Accordingly, PKCζ was coprecipitated with active RAC1, with the amount decreased in CEBPγ-knockdown cells. Knocking down of YBX1 was associated with rather increase in the RAC1 and PKCζ, suggesting that the regulation through YBX1 is not simple. These results indicate that both lamellipodia formation and migration activity are, at least partly, dependent on CEBPγ expression. Furthermore, YBX1, may also positively regulate lamellipodia formation but not migration activity.

In order to determine whether CEBPγ, YBX1, and the putative downstream factor CERS6 promote cancer cell migration in patients, a clinical dataset containing information for 90 adenocarcinoma patients was analyzed. Those results showed that the expression level of each of

those genes was significantly associated with degree of invasiveness (Figure 5A). We also analyzed the expressions of CERS6 and YBX1 proteins in clinical patients. YBX1 expression was observed in all 20 of the clinical lung cancer specimens, with expression patterns homogeneous in 14. Interestingly, in the other 6 cases, the YBX1 patterns were heterogenous and similar to those of CERS6 varied among the patients (Figure 5B). These results led us to speculate that YBX1 is one of the positive regulators of CERS6 expression.

【Discussion and conclusion】

Ceramide synthase 6 (CERS6) expression has effects on cellular ceramide constitution to upregulate the d18:1/16:0 ceramide (C16 ceramide) level, resulting in stimulation of cell migration and invasion activities through RAC1-positive lamellipodia formation. Our results demonstrate that the transcription factor CEBPγ promotes CERS6 expression via specific interaction with Y-box, and that it also regulates lamellipodia formation and migration activity. YBX1 may also be involved in this pathway (Figure 6). This study provides novel insights into transcriptional regulation mechanisms of CERS6 in lung cancer.