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学位の種類	博士（医学）
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審査研究科	人間総合科学研究科
学位論文題目	Glycoprotein NMB functions with growth factor signaling to induce tumorigenesis in breast cancer cells（GPNMB は増殖因子シグナルと協調して乳がん細胞の腫瘍形成を促進する）

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論文の内容の要旨
Abstract of thesis

In this doctoral dissertation, the author describes characterization of GPNMB (Glycoprotein non-metastatic gene B) protein phosphorylation and its significance in tumorigenesis. GPNMB has been studied as a tumorigenic factor in breast epithelial cells which works by modulating epithelial-mesenchymal transition (EMT). The author identified Ser530 on human GPNMB as a conserved potential phosphorylation target within the KGLS motif. The author detected Ser530 phosphorylation by mass-spectrometry (MS) analysis, and raised specific monoclonal antibodies. Using these antibodies, Ser530 phosphorylation was verified in 293T cells and breast cancer cells MCF-7, Hs578 and NMuMG. The author then examined the biological function of Ser530 phosphorylation using alanine-substitution mutants. Results from cell-lines expressing GPNMB Ser530 to Ala substitution mutant indicate that GPNMB phosphorylation is important for sphere formation and expression of stemness markers in 2D and 3D culture systems. With these results, the author proposed that Ser530 phosphorylation is important for tumorigenic function of GPNMB through EMT-inducing properties. The content is summarized as follows:

（目的　Purpose）

The purpose of this research is to characterize phosphorylation of GPNMB and to examine its functional significance.

（対象と方法　Materials and Methods）

The author raised monoclonal antibodies against phosphor-GPNMB. Monoclonal antibodies were produced by Ser530-phosphorylated synthetic peptide antigen immunization and subsequent hybridoma production and screening. To further verify this phosphorylation event, the author examined phosphorylation status of purified FLAG-GPNMB expressed in 293T cells by tandem-chromatography MS/MS. Stable cell-lines for wild-type and serine to alanine substitution mutant GPNMB(SA) were produced using FLAG/HA-tag vector and transfection in MCF7 and NMuMG cells. RNA expression of stemness and EMT marker genes were quantified by real-time PCR. Transwell migration assay was performed by a commercial chamber. Sphere formation assay was performed using commercially available low-adhesion culture plate. Tumor formation assay was based on cell injection to 6-week-old Balb/c mice and tumor volume examination 8 weeks after injection. Pathway analysis of RNAseq results was performed against DAVID and KEGG databases.

（結果　Results）

The author predicted potential phosphorylation sites based on both conservation and phosphorylation site prediction by eukaryotic linear motif (ELM) database. Using phospho-peptide immunization, monoclonal antibodies were raised and verified by Western blot and immunofluorescence staining of cells. These antibodies were further characterized using GPNMB-expressing cell-lines, and their specificity was confirmed. The GPNMB(SA) overexpressing cells showed the significant reduction of sphere formation in vitro and tumor growth in vivo. GPNMB(SA) cells showed less stemness-related genes expression compared to those in GPNMB(WT) cells based on RNA expression analysis. Additionally, the GPNMB(SA) mutation impaired GPNMB-mediated cellular migration. Tyrosine kinase receptor signaling triggered by EGF or FGF-2 induced the phosphorylation of Ser530 through activation of downstream oncoproteins, RAS and RAF.

（考察　Discussion）

In this study, the author demonstrated phosphorylation of Ser530 in human GPNMB and conserved site in mouse. It was previously shown that tyrosine residues in the GPNMB intracellular domain are important for its tumorigenic activity. Therefore, the author’s findings add significant value to the understanding of GPNMB post-translational modification by previously uncharacterized Ser phosphorylation. Previously, it was reported that GPNMB was expressed in a variety of normal tissues, such as bone, skin and hematopoietic system, and affected cell proliferation, adhesion, and differentiation. The author established the role of Ser530 phosphorylation in stemness-related cell behavior and tumor formation in mice. The results also showed that EGF and FGF-2 induces phosphorylation of GPNMB Ser530 through activation of RAS and RAF, but not of MEK and ERK. Since the receptor-tyrosine kinase pathway is critical in regulating cell proliferation, differentiation, and survival, these results indicate phosphorylation of Ser530 as a key modification for GPNMB-medicated tumor formation by EMT induction and increased stemness.

審査の結果の要旨
Abstract of assessment result

（批評　General Comments）

The applicant examined phosphorylation status of Ser530 on GPNMB protein by MS and by developing new antibodies in multiple cell-lines. These analyses established phosphorylation status of conserved serine residue in the intracellular domain of GPNMB. Further characterization of functional significance of this phosphorylation site revealed its significance in EMT and critical role in tumorigenicity.

（最終試験の結果　Assessment）

The final examination committee conducted a meeting as a final examination on September 28, 2021. The applicant provided an overview of dissertation, addressed questions and comments raised during Q&A session. All of the committee members reached a final decision that the applicant has passed the final examination.

（結論　Conclusion）

The final examination committee approved that the applicant is qualified to be awarded Doctor of Philosophy in Medical Sciences.