論文の内容の要旨

論文題目 **A NOVEL *SOS1* MUTATION IN COSTELLO/CFC OVERLAPPING SYNDROMES ENHANCES DOWNSTREAM SIGNAL IN RAS/MAPK PATHWAY**
(コステロ/CFC 重複症候群の新規 *SOS1* 遺伝子変異は下流の RAS/MAPK 経路シグナルを増強する)

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PART I. A NOVEL *SOS1* MUTATION IN COSTELLO/CFC OVERLAPPING SYNDROMES ENHANCES DOWNSTREAM SIGNAL IN RAS/MAPK PATHWAY

INTRODUCTION: The RAS/MAPK pathway is activated by guanine nucleotide exchange factors (GEFs). SOS1, the major GEF factor, and activated GTP-bound RAS by SOS1 interact with over twenty effector proteins: among the best characterized are RAS kinases and phosphatidylinositol 3-kinase (PI3K). RAS activates RAF, and activated RAF activates mitogen activated protein kinase kinase (MEK). Activated MEK, in turn, activates ERK1/2. RAS also binds directly to the p110 subunit of PI3K. As with RAF, several lipids and kinases can activate PI3K. Previous studies have revealed roles of the RAS/MAPK in cell proliferation and differentiation, but underlying signaling network with PI3K/AKT, the second major pathway remains unclear. It is known that PI3K/AKT signaling cooperatives with RAS signaling in regulating growth factor-stimulated cell cycle progression. Recently, germline mutations of genes of RAS/MAPK pathway have been reported in neuro-developmental disorders including Noonan, Costello and Cardio-facio-cutaneous (CFC) syndrome. All these mutations are known to cause gain of function of the genes.

In a previous study, I found a novel mutation of the *SOS1* gene, T158A, in a patient showing an exceptional phenocopy of Costello syndrome. This mutation was located in histone like fold domain. SOS1 contains a RAS-GEF (Cdc25) domain, as well as conserved histone-like fold, Dbl homology and pleckstrin homology domains, a helical linker, a RAS exchange motif and a proline-rich region. Alteration of most of these domains is known or predicted to disrupt autoinhibition of SOS1 RAS-GEF activity. *In vitro* assays showed that mutations in Dbl, helical linker, Cdc25 and pleckstrin domains of *SOS1* show increased EGF-evoked ERK activation, confirming gain of function of *SOS1* in Noonan syndrome. Up to now, little is known about function of the histone-like fold domain of SOS1 in auto-inhibition role for CDC25 domain.

OBJECTIVE: The objective of this study is to investigate whether novel *SOS1* T158A can activate RAS/MAPK pathway in Costello/CFC syndrome.

MATERIALS AND METHODS: Human *SOS1* was used as a template. The primers were designed to amplify target mutation (Forward: SOS1Mut1F: ATATACGGCATTATGAAATTGCAAAACAAGATAT and Reverse: SOS1Mut1R: AATTTCATAATGCCGTATATTTCTTACATAAT). HEK293T cells were transfected with wild type and mutant SOS1 T158A plasmid. After 4 hours of transfection with Multifectam transfection kit, cells were cultivated for 24 hours, then starved for 18 hours and EGF (100ng/ml) was added for 5, 15 and 30 min. In another experiment, cells were added with EGF for 20 min and incubated continuously in serum free meduim for 18 hours. The primary antibodies were anti-phospho-

ERK1/2 kinase (Thr202), phospho-AKT(Ser 473), and phospho-S6 (Thr389), total ERK1/2, AKT and S6, and anti-SOS1. Phosphorylated ERK1/2 to total ERK1/2 ratios was quantified using the Image J software and *t* test was used for statistics. Ratios of phosphorylated S6 and AKT to total S6 and AKT were also analyzed.

RESULTS: Although the basal level of phosphorylated ERK1/2 was low both in transfected and non-transfected cell lines, it increased rapidly after EGF treatment. The ratio of phospho-ERK1/2 to total ERK1/2 was significantly higher at 5 min after EGF stimulation in mutant SOS1 cells than in wild type SOS1 cells. In the wild SOS1 cell lines, the ratio reached its peak after 15 min of EGF stimulation. Phospho-AKT was less detectable in wild type SOS1 cells at basal condition, as well as at 5 and 15 min after stimulation. At 30 min after stimulation, it became clearly detectable, and was less abundant in mutated SOS1 cells than in wild type SOS1 cells. The increase of phosphorylated ribosomal S6 was more prominent and earlier in mutated SOS1 cells than in wild type SOS1 cells.

When cells were starved for 18 hours after EGF stimulation, the level of phospho-ERK1/2 in mutated SOS1 cell lines was significantly higher ($p=0.03$) than in wild type SOS1 cells, suggesting a continuous activation of mutant SOS1 leading to excessive RAS activity in response to growth factor stimuli. There was no difference in the expression level of phospho-AKT at 18 hours after EGF stimulation. These results demonstrate that, under stimulation by a growth factor, *SOS1* T158A mutation causes excessive and sustained activation of the RAS/MAPK pathway.

DISCUSSION: The T158A, mutation of the *SOS1* gene located in its histone-like fold domain, has never been reported in Costello, Noonan and CFC syndromes. It prompted us to carry out functional study to characterize this mutation, and to assess whether the mutated protein cause Costello/CFC syndrome by activating RAS/MAPK pathway. In this study, I have elucidated that, T158A mutation indeed causes hyper-activation of ERK1/2 in RAS/MAPK pathway. Expression of T158A resulted in constitutive RAS activation. Induction by EGF elicited a stronger response, both in magnitude and duration, indicating the functional consequence of the *SOS1* mutation in 293T cells. These results confirmed our predictions that CFC/Costello associated T158A mutation in histone-like fold will abrogate autoinhibition of SOS1 protein, thereby resulting in increased downstream signaling of RAS/MAPK pathway.

PI3K activates the protein kinase PDK1 which in turn activates the serine/threonine kinase AKT. This study showed that SOS1 protein functions for both RAS and PI3K, leading to ERK and AKT dysregulation. The T158A mutant showed a transient increased activation of AKT in EGF stimulated 293T cells. In the downstream of both ERK1/2 and AKT, there is p70 S6 kinase, a mitogen activated Ser/Thr protein kinase that is an activator of phosphorylation of ribosomal S6. ERK1/2 and S6 phosphorylation was higher activated in mutant SOS1 cells than in wild type SOS1 cells. The P13K/AKT signaling pathway was activated by EGF both in mutant and wild type SOS1 cells however, AKT activation was smaller induced in mutant SOS1 cells. There is a feedback mechanism in which p70 S6 kinase phosphorylates insulin receptor substrate-1 to inhibit PI3K and AKT activation. Thus, in mutant SOS1 cells, excessive p70 S6

kinase may inhibit AKT pathway. In conclusion, combined dysregulation of RAS and AKT activities may cause overlapping syndromes of RAS/MAPK pathway. To normalize both RAS and AKT signaling should be an essential component of successful treatment strategies.

***CONCLUSION*:** The *SOS1* mutation T158A causes gain of function leading to an increase in phosphoralytion of ERK1/2, responsible for CFC/Costello phenotype. Combination of RAS/MAPK pathway and PI3K/AKT pathway may account for variable clinical expression of overlapping syndromes of RAS/MAPK pathway.

PART II. MOLECULAR GENETIC ANALYSIS OF OVERLAPPING SYNDROMES OF THE RAS/MAPK PATHWAY

INTRODUCTION: Genes of RAS/MAPK pathway are well studied because they are frequently activated in human cancers and play a pivotal role in cell proliferation, differentiation, survival, and cell death. Very recently, in a surprising development, mutations in several RAS pathway members have been identified in patients with multiple congenital malformation syndromes: mutations of *PTPN11* and *KRAS* genes Noonan syndrome (33-60% and 1% of patients, respectively), those of *BRAF*, *KRAS* and *MEK1/2* in CFC (78%, 7% and 12% of patients, respectively) and those of *HRAS* in Costello syndrome (87%), establishing a new role of the RAS/MAPK pathway in human development. However, causative genes remain unknown in many patients with these disorders.

MATERIALS AND METHODS: We recruited four patients with diagnosis of Noonan syndrome and one with Noonan/Costello case from Japan, and thirteen Noonan patients from Mongolia. The purpose of this study was to detect mutation of associated genes for RAS overlapping syndromes. After informed consent, peripheral blood samples from all patients were collected and genomic DNA was extracted using standard protocols. For control subjects, we recruited 50 healthy volunteers. I analyzed entire coding regions of *PTPN11, KRAS, HRAS, RAF1*, and *NRAS* and exons of *BRAF, SOS1* and *MEK1*, where mutations have previously been reported. Deleted nucleotide was confirmed by subcloning.

RESULTS:

1. A Noonan/Costello case from Japan had a heterozygous mutation in exon 7 of *RAF1* gene. The C to T transition at position 770 predicted serine to leucine substitution within the CR2 domain. This mutation has previously been reported in Noonan patients with hypertrophic cardiomyopathy.
2. A Noonan case from Mongolia had a heterozygous mutation in exon 3 of *MEK1* gene. The G to A transition at position 338 caused arginine to lysine substitution in the protein kinase domain. The mutation has never been reported in Noonan, CFC or Costello syndrome, and was not found in healthy 50 controls.
3. Another Noonan case from Mongolia had a single base deletion, IVS8-19delA of *SOS1* gene.
4. Three Noonan cases from Japan and one Noonan case from Mongolia had a minor allele A (rs4362222) in exon 5 of *KRAS* gene.

5. The remaining fifteen Noonan patients including Japanese and Mongolian cases did not have *PTPN11* mutations. They had no mutations in *SOS1, BRAF, RAF1, MEK, KRAS, HRAS* and *NRAS* genes.

***DISCUSSION*:** I found a novel mutation of *RAF1* gene, causing an amino acid substitution of serine to leucine at position of 257 within domain of CR2, in a Noonan/Costello case. The Noonan patients with S257L reported previously were all diagnosed in their young age (3-8 years) because of their high prevalence of skin features (71%) and hypertrophic cardiomyopathy (100%). The present case also had hypertrophic cardiomyopathy. However, her clinical features resembled Costello syndrome rather than Noonan syndrome, suggesting a complex genotype-phenotype correlation in these syndromes.

A Mongolian Noonan case had novel mutation of Arg113Lys in *MEK1,* which has never been reported before. This mutation site was highly conserved in vertebrates and was predicted to be probably damaging in online software (SIFT). *MEK1* mutations are reported in 20% of CFC patients, but are uncommon in Noonan patients. This patient's clinical features showed Noonan phenotype.

Another Mongolian Noonan case had a single nucleotide deletion in intron 8-9 of *SOS1* gene, which has never been reported in Noonan, CFC and Costello syndrome. Intronic sequences that lie outside the consensus splice site signals and branch site have been implicated in affecting splicing events (Goldstrohm et al. 2001). Notably, the mother of this case was a typical Noonan patient. The deletion site is possible binding site of hnRNP (http://www.intron i.it/splicing.html). Therefore the deletion might affect the function of hnRNP and splicing pattern of *SOS1* in this patient. To confirm pathological significance, it is necessary to elucidate the alternative splicing of *SOS1*. Up to now, frame shift insertion in *SOS1* has been reported in one patient with hereditary gingival fibromatosis.

Three Japanese Noonan cases and one Mongolian Noonan case had a minor allele A in exon 5 of *KRAS* gene (rs4362222). None of 120 Japanese and Han Chinese control subjects has been reported with this single nucleotide variation (SNV) in online database (http://www.ncbi.nlm.nih.gov/SNP/). No information about Mongolian control subjects is available so far. Software analysis showed this SNP is an exonic splicing enhancer or silencer which means it may have functional effects by disrupting splice site. (http://snpinfo.niehs.nih.gov/snpfunc.htm). This SNV has been reported in patients with Noonan cases and those with lung cancers and mental retardation. Pathologic significance of this variant remains to be elucidated.

CONCLUSION: I found a *RAF1* mutation that is commonly associated with Noonan syndrome, in a patient with overlapping Noonan/Costello phenotype. I did genetic analysis in thirteen Mongolian Noonan patients for the first time, and found a novel amino acid substitution in *MEK1* gene and a single nucleotide deletion in *SOS1* gene. A rare SNP in *KRAS* gene was found in four patients including Japanese and Mongolian cases. These exceptional cases again highlight the complex relationship between the genotype and phenotype of overlapping syndromes of the RAS/MAPK pathway.