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１．研究テーマ

HNF異常型糖尿病発症機構の解明及び治療の開発

２．本年度の研究業績

(1) 学会・研究会等における発表　有　・　無　（学会名・演題）

(2) 学会誌等に発表した論文　有　・　無　（雑誌名・論文名）

T130 mutation in HNF-4α gene is a loss-of-function mutation and is associated with late-onset type 2 diabetes in Japanese subjects
(準備中)

日中医学協会助成事業

HNF-4 α 遺伝子 T130I 変異と日本人２型糖尿病の関係を検討いて

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Abstract:

The T130I mutation in HNF-4α is a missense mutation and it was suggested this mutation cosegregated with typical late-onset NIDDM. From screening for T130I mutation, we found that the frequency of the mutation was significantly higher (P=0.015) in the type 2 diabetic group compared with nondiabetic subjects (15 out of 423 diabetic subjects and 3 out of 354 nondiabetic subjects).From clinic data, we found the serum HDL-cholesterol level was significantly lower in the group with the T130I mutation. Next, we found that the transcriptional activity of T130I -HNF-4α was significantly decreased by 46.2%compared with that of WT-HNf-4α in HepG2 cells and human hepatoma cell line, but the difference of the transcriptional activity between T130I -HNF-4α amd WT-HNf-4α was not significantly in HeLa cell and MIN6 cell. line. And the binding of T130I -HNF-4α relative to WT-HNf-4α was similar under both equilibrium and non-equilibrium conditions. More studies and HNF-4α knockout mouse models will be necessary to provide additional insights into the molecular mechanism.

Key Words: HNF-4α NIDDM serum HDL-cholesterol level human hepatoma cell line

Introduce:

MODY is a monogenic form of type 2 diabetes characterized by autosomal dominant inheritance, early-onset (usually before 25 years of age), and impaired insulin secretion. Mutations in the HNF-4α gene cause a form a MODY (MODY1). Recent studies obtained the nominal evidence for linkage of markers in the region of the HNF-4α gene on chromosome 20q12-q13 with NIDDM. And a genetic variation in the HNF-4α cosegregated with typical late-onset NIDDM. The T130I mutation is a missense mutation affecting a residue of the DNA- binding region (A-box region). In Danish and Japanese study, the frequency of this allele was significantly higher in individuals with type 2 diabetes than in control subjects. In this study, we examine the diabetogenic impact of the T130I mutation by emplying genetic and functional analyses.

Subjects and Methods:

Subjects: We screened 423 unrelated Japanese subjects with type 2 diabetes and 354 unrelated Japanese nondiabetic control subjects for T130I mutation in HNF-4α gene. Exon 4 and flanking intron were amplified-using PCR. T130I mutation generates a BsmI site and it was detected by PCR-restriction fragment length polymorphism.

Plasmid: T130I mutant HNF-4α was generated from human HNF-4α 2 cDNA using a Chameleon Double –Stranded Site-Directed Mutagenesis Kit (stratagebem Jolla, CA) and cloned in PcDNA3.1 expression vector (Invirogen, San Diego, CA). The construct was tested by DNA sequencing.

Cell culture and Luciferase assay: Mouse primary hepatocyte were prepared using collagenase perfusion method and plated in six-well tissue culture plates. HeLa, MIN6, HepG2 cell and primary hepaocytes were transfected with 500ng expression and reporter vectors together with 10ng of pRL-TK (promega, Madison, WI) as an internal control, using LIPOFECTAMIN PLUS reagent. Transactivation activities were measured after 48 hours using the Dual Luciferase Reporter Assay system.

Western blot analysis and electrophoretic mobility shift assay (EMSA): Western blot analysis was performed using anti-HNF-4α antibody (Santa Creuz Biotechnology, Santa Cruz, CA) as described previously. Wild type and T130I-HNF-4α proteins were synthesized using TNT T7 Quick Coupled Transcription/Translation System (Promega). In viro translated proteins were incubated with 32P-labeled oligonucleotides containing HNF-4α binding site of HNF-1α gene in a 20-υl-reaction mixture. DNA-protein complexes were analyzed on 5% polyacrylamide gels using 0.5X TBE buffer. The polyclonal anti-HNF-4αantiserum was used for supershift analysis.

Result:

First, T130I mutation was found in 15 out of 423 diabetic subjects (3.4%) and in 3 out of 354 nondiabetic subjects (0.8%), indicating that the frequency of the mutation was significantly higher (p=0.015) in the type 2 diabetic group compared with the nondiabetic Japanese subjects. The clinical features of the diabetic subjects with the Ile codon at 130 are shown in Table1. The average age at diagnosis of diabetes with MODY1 was 28.2 ± 15.2 years. The mean age at diagnosis was significantly higher for patients with the T130I mutation (47.1 ± 8.8 years, p=1.0x106) than for those with other HNF-4α mutations. The serum HDL-cholesterol level was significantly lower in the group with the T130I mutation.

Next, we investigated the function of T130I –HNF-4α. HeLa cells and MIN6 cells were transfected with wild-type (WT) HNF-4α or T130I HNF-4α expression. The levels of WT and T130I-HNF-4α expression were similar. (Fig. 1A 1B and 1C). However, when the same amount of expression vector was

transfected into HepG2 cells, a human hepatoma cell line, the transcriptional activity of T130I-HNf-4α was significantly decreased by 46.2%(P<0.001) compared with that of WT-HNF-4α (Fig 1D). Figure 1E shows that impaired transactivation of T130I-HNF-4α in HepG2 cells were found at all doses tested, while T130I-HNF-4α achieved similar transactivation compared with Wt-HNF-4α in MIN6 cells at these doses (data not shown). Reduced transactivation of T130I-HNF-4α (27.9%of WT-HNF-4α , P=9.7x10-5)was also found in primary hepatocytes (Fig . 1F). HNF-1α and L-type pyruvate kinase (PKL) are target genes for HNF-4α. Transcriptional activation of the HNF-1α gene (78.2%, P=0.024) and PKL gene (77.1%, P=0.002) by T130I-HNF-4α was impaired (Fig. 1G). these data strongly suffest that T130I-HNF-4α cats as a loss-of-function mutation in hepatic cell environment.

Since the A-box region is considered to be important for DNA binding, we also tested the DNA binding ability of T130I-HNF-4α . WT-HNF-4α and T130I-HNF-4α specifically bound to the oligonucleotide (Fig 2A). The binding of T130I-HNF-4α relative to WT-HNF-4α was similar under both equilibrium and non-equilibrium conditions. (Fig 2B), suggesting that this amino acid change does not later DNA binding at least in virto. At present, it is not clear why the T130I mutation only affects transactivation activity in hepatic cells. Further studies will be necessary to clarify the mechanism involved.

Conclusion:

In this study, we demonstrated that T130I mutation in HNF-4α gene was associated with late-onset type 2 diabetes in Japanese individuals and found that this loss-of-function mutation, at least in hepatocytes. The molecular mechanism by which low HNF-4α activity in hepatocytes, but not in pancreatic β-cells, leads to late-onset diabetes is unknown at present. More studies and HNF-4α liver knockout mouse models will be necessary to provide additional insights into the molecular mechanism.

Figure1.Transactivation activities of wild type (WT) and T130I-HNF-4α

A- C: Transactivation activities of WT and T130I-HNF-4α in HeLa (A), MIN6 (B). and HepG2 (C) cells. Cells were transfected with 500ng of expression vectors together with 100ng of pHNF4-tk-Luc and 10ng of pRL-TK. D:Transactivation activities of T130I-HNF-4α in HepG2 cells. Increasing amounts of expression vectors (50-200ng) were transfected with pHNF-4–tk-Luc. Transcriptional activity of T130I-HNF-4α was impaired in HepG2 cells. Data are mean ± SD values of three independent experiments. E-G: Mouse primary hepatocytes at a density of 3x105 cells/well were transfected with expression vectors together with 100ng of reporter genes. (E:pHNF-4-tk-Luc;F:HNF-1α promoter;G:PKL promoter). Data are mean ± SD values of six independent experiments. H: Expression of WT and T130I-HNF-4α in HeLa cells. Eight ug of expression vectors were transfected and Western blot was performed after 48 hours.

Figure 2. DNA binding ability of WT and T130I-HNF-4α .A: HNF-4αproteins were in vitro translated from 1ug of pcDNA3.1 expression vector using TNT in virto transcription/translation system and used for EMSA. Equal expression levels of in vitro translated proteins were confirmed by Western blot analysis (data not shown). An excess (50-fold) of unlabed oligonucleotide was used as a competitor. Lane7and8 show supershift of bands. B: cDNA binding ability of T130I-HNF-4α in non-equilibrium conditions. WT-HNF-4α and T130I-HNF-4α were bound to the labeled oligonucleotide in the presence of increasing amount (10, 25 and 50 molar excess) of unlabeled competitor in non-equilibrium conditions.

本文は投稿中です。

作成日：２００３年３月１５日

TABLE 1
Clinical characteristics of the type 2 diabetic subjects with and without T130I-HNF-4α mutation

Characteristic	Thr/Thr	Thr/Ile	P
n	408	15	
M/F	245/163	8/7	
age (years)	64.0 ± 9.0	61.5 ± 6.3	NS
age at diagnosis of diabetes (years)	47.7 ± 8.2	47.1 ± 8.8	NS #28.2 ± 15.2 (n=40) ($p=1.0x10^{-6}$**)
BMI (kg/m^2)	22.9 ± 3.1	24.3 ± 3.6	NS
maximum BMI (kg/m^2)	26.2 ± 3.5	27.3 ± 3.6	NS
FPG (mg/dl)	151 ± 47	153 ± 44	NS
HbA1c (%)	7.6 ± 1.6	7.2 ± 1.4	NS
HOMA-IR (mol mU/l^2)	2.4 ± 1.8 (n=134)	2.2 ± 1.1 (n=8)	NS
T-chol (mg/dl)	202 ± 36 (n=244)	195 ± 39 (n=9)	NS
triglyceride (mg/dl)	139 ± 119 (n=244)	158 ± 75 (n=9)	NS
HDL-chol (mg/dl)	55 ± 18 (n=239)	40 ± 13 (n=9)	0.006**

Data are means ±SD. #Age at diagnosis of MODY1 subjects (data are cited from references 14-22). HOMA-IR =FPG x fIRI/22.5. P values were obtained by the two-tailed Student's t test. **P<0.01. NS; not significant.

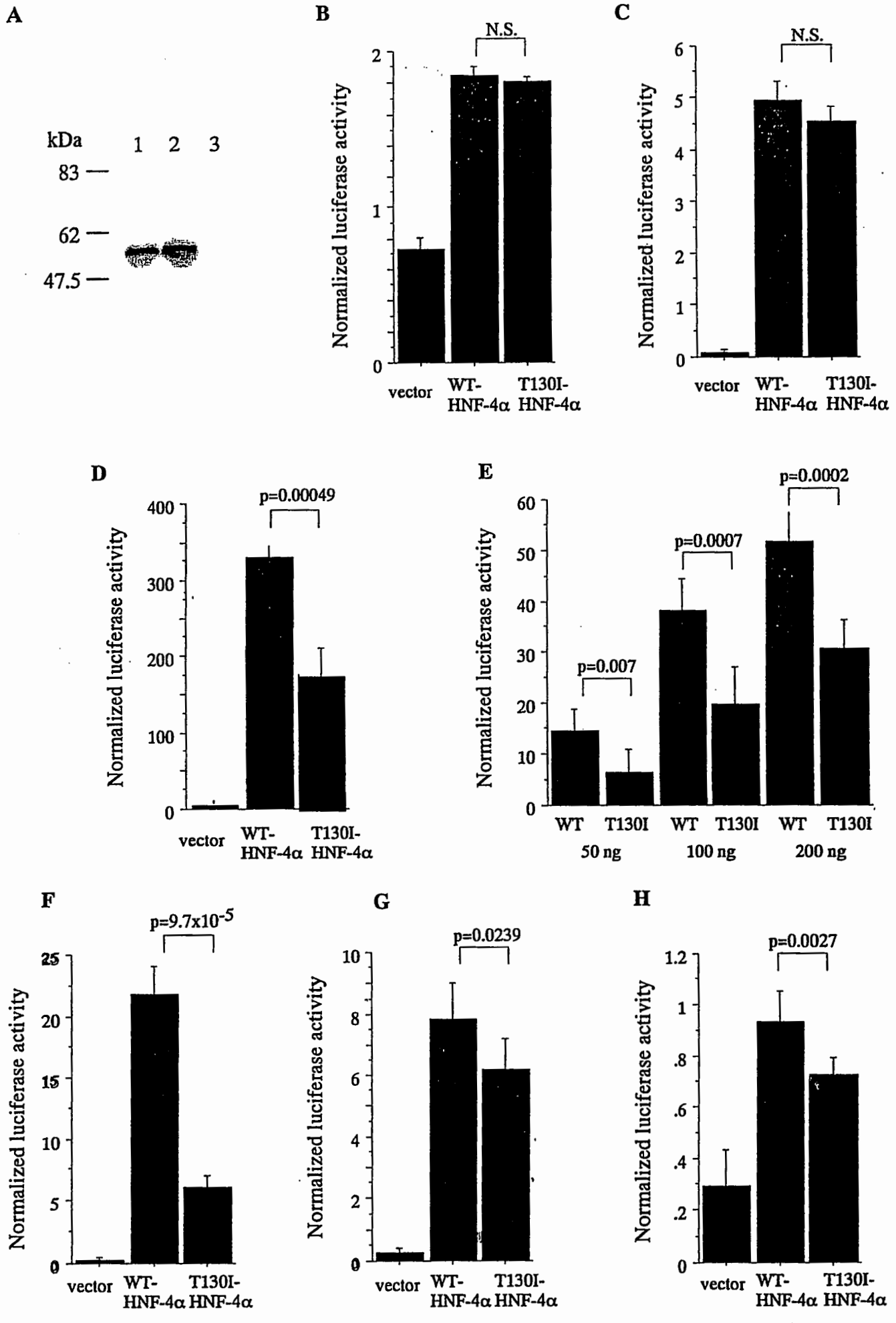
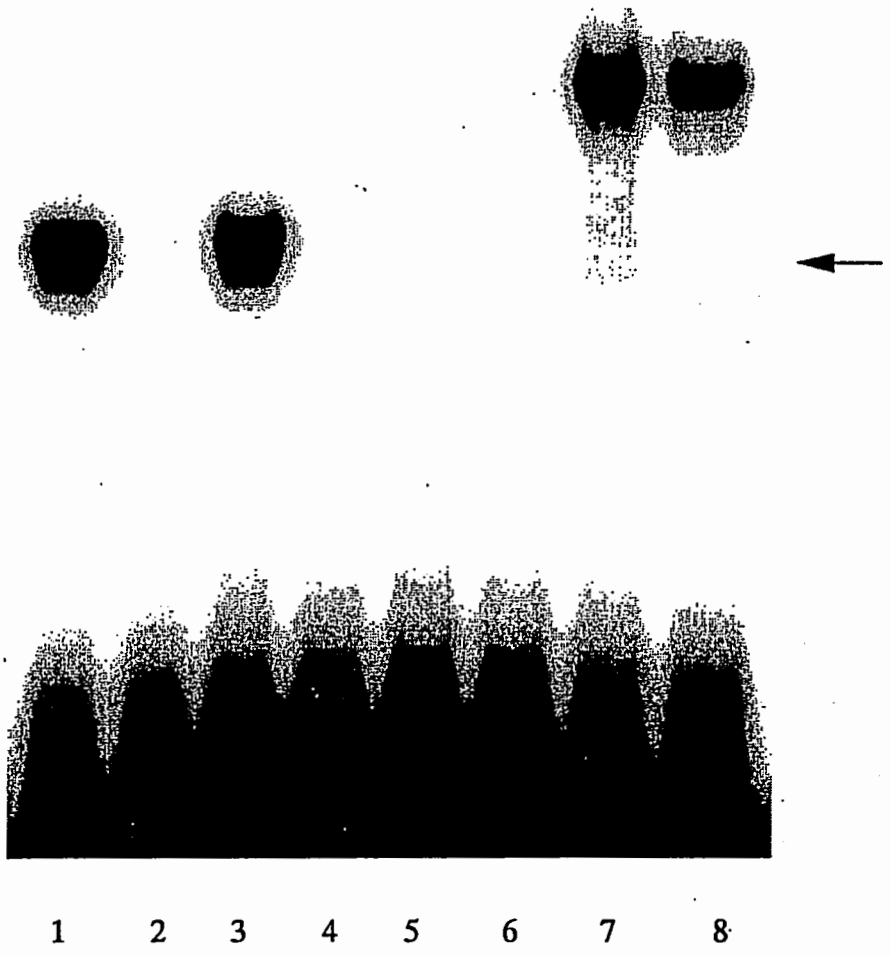

A
kDa
1 2 3
83
62
47.5
B
N.S.
Normalized luciferase activity
vector
WT-HNF-4α
T130I-HNF-4α
C
N.S.
Normalized luciferase activity
vector
WT-HNF-4α
T130I-HNF-4α
D
p=0.00049
Normalized luciferase activity
vector
WT-HNF-4α
T130I-HNF-4α
E
p=0.0002
p=0.0007
p=0.007
Normalized luciferase activity
WT T130I WT T130I WT T130I
50 ng 100 ng 200 ng
F
p=9.7x10-5
Normalized luciferase activity
vector
WT-HNF-4α
T130I-HNF-4α
G
p=0.0239
Normalized luciferase activity
vector
WT-HNF-4α
T130I-HNF-4α
H
p=0.0027
Normalized luciferase activity
vector
WT-HNF-4α
T130I-HNF-4α

Figure 1

A



1 2 3 4 5 6 7 8

B

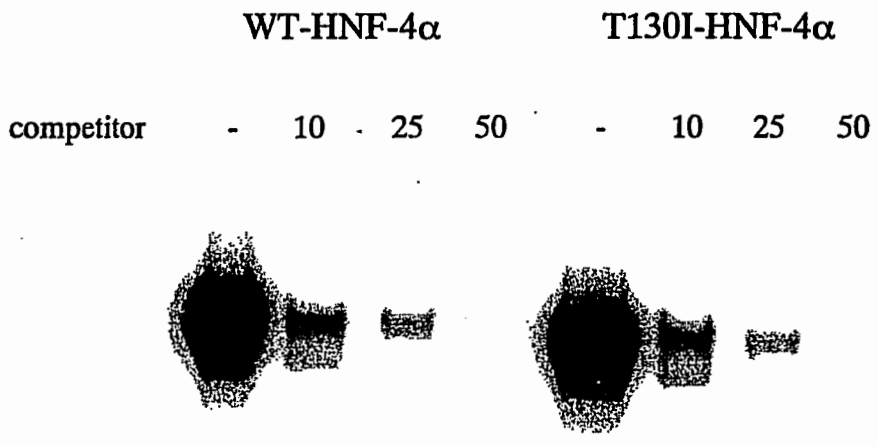


Figure 2