*Short communication***R127W-HNF-4 α is a loss of function mutation but not a rare polymorphism and causes Type II diabetes in a Japanese family with MODY1****Q. Yang¹, K. Yamagata¹, K. Yamamoto², Y. Cao¹, J. Miyagawa¹, A. Fukamizu³, T. Hanafusa¹, Y. Matsuzawa¹**¹ The Department of Internal Medicine and Molecular Science, Graduate School of Medicine, Osaka University, Japan² The Department of General Medicine, Osaka University Hospital, Osaka, Japan³ Centre for Tsukuba Advanced Research Alliance, Institute of Applied Biochemistry, University of Tsukuba, Ibaraki, Japan**Abstract**

Aims/hypothesis. Mutations in the hepatocyte nuclear factor (*HNF*)-4 α gene cause the type 1 form of maturity-onset diabetes of the young (MODY1). The R127W mutation is a missense mutation located in the T-box region of HNF-4 α that was first identified in a Japanese family with MODY. We have examined the functional properties of this mutation in order to clarify the molecular basis of MODY1.

Methods. The intracellular localisation, DNA binding ability, transactivation activity and functional synergism with the coactivator CREB-binding protein (CBP) of R127W-HNF-4 α were investigated.

Results. The nuclear import and functional synergy with CBP of R127W-HNF-4 α were normal. The DNA binding ability of the mutant was decreased as was its transcriptional activation of the *HNF-1 α* and L-type pyruvate kinase (*PKL*) genes.

Conclusion/interpretation. The R127W mutation seems to be a loss-of-function mutation. [Diabetologia (2000) 43: 520–524]

Keywords MODY, HNF-4 α , HNF-1 α , L-type pyruvate kinase, insulin.

Maturity-onset diabetes of the young (MODY) is a monogenic form of diabetes mellitus characterised by autosomal dominant inheritance and early onset. Mutations in the hepatocyte nuclear factor (*HNF*)-4 α gene cause MODY1 [1]. Hepatocyte nuclear factor-4 α is a transcription factor and a member of the steroid hormone receptor superfamily. It consists of several functional domains: an N-terminal transactivation domain (AF-1), a DNA binding domain followed by a T-box and an A-box and a functionally

complex C-terminal region that forms a ligand binding domain, a dimerisation interface and a transactivation domain (AF-2) [2]. It plays an important part in the regulation of genes involved in a variety of metabolic pathways. The expression of the *HNF-1 α* /*MO Δ W3* gene is also regulated by HNF-4 α .

The R127W mutation is a missense mutation identified in a Japanese family [3]. This mutation changes a conserved arginine located in the T-box (amino acids 117–128), a region which is suggested to be important for dimerisation and DNA binding [2]. Hepatocyte nuclear factor-4 α has been shown to be coactivated by CREB (cAMP-responsive element binding protein) binding protein (CBP) [4]. The region including the arginine residue at 127 has been reported to be critical for the interaction with CBP [4]. Thus, we hypothesised that the R127W mutation impairs the functional activity of HNF-4. Recently the transactivation activity of R127W-HNF-4 α has, however been reported to be normal [5]. Here we show that the transcriptional activation of the *HNF-1 α* and L-type pyruvate kinase (*PKL*) promoters is impaired

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Abbreviations: MODY, maturity-onset diabetes of the young; CBP, cAMP-responsive element binding protein; PKL, L-type pyruvate kinase; WT, wild-type; TK, thymidine kinase; EMSA, electrophoretic mobility shift assay; HNF, hepatocyte nuclear factor; PI, propidium iodide.

by R127W-HNF-4 α . The decreased expression of *HNF-1 α* and *PKL* genes could contribute to the beta-cell dysfunction that characterises HNF-4 α diabetes.

Methods

Plasmid constructs. We generated R127W and Q268X-HNF-4 α cDNAs using the Chameleon Double-Stranded Mutagenesis Kit (Stratagene, La Jolla, Calif., USA) and subcloned them into pcDNA3.1 (Invitrogen, Carlsbad, Calif., USA). The nucleotide sequence encoding the FLAG epitope (DYKDDDDK) was introduced in the frame at the C-termini of the wild-type (WT) and R127W sequences. The CD1b-HNF-4 α mutant is a C-terminal truncated mutant retaining the DNA binding ability [6]. It was generated as described previously [6]. Human HNF-1 α gene promoter (nucleotides -129 to 196) was subcloned into pGL3-basic vector (Promega, Madison, Wis., USA). The promoter region between positions -206 and -7 of the rat PKL gene including the HNF-4 α binding site was amplified by PCR and subcloned into pGL3-basic vector. The sequence of each construct was verified. We inserted eight copies of the HNF-4 α binding site of the mouse transthyretin promoter upstream of the HSV-TK promoter/luciferase fusion gene (pHNF4-tk-Luc). The CBP expression vector, pRc/RSV-CBP, has been described previously [4].

Cell culture and transient transfection assays. We maintained HeLa and MIN6 cells in D-MEM supplemented with 10% and 15% fetal bovine serum, respectively. We used MIN6 cells in experiments between passages 7 to 12. When reaching 80% confluence, HeLa and MIN6 cells were detached and plated in 6-well plates at a density of 1.5×10^5 (HeLa) or 3×10^5 (MIN6) cells per well. Cells were transfected for 3 h in serum-free OPTI-MEMI (Life Technologies, Rockville, Md., USA) with the indicated amounts of the expression vectors (Fig. 2 legend) and 0.5 μ g of the reporter genes together with 10 ng of pRL-SV40, the internal control plasmid (Promega), using the LipofectAMINE PLUS Reagent (Life Technologies) according to the manufacturer's protocol. After another 48-h incubation, luciferase activity was measured.

Immunolocalisation of HNF-4 α in transfected cells. The HeLa cells (1×10^5) grown on Lab-Tek chamber slides (Nalge Nunc, Naperville, Ill., USA) were transfected with 0.5 μ g of FLAG-epitope-tagged WT and R127W-HNF-4 α constructs. After 48 h, cells were fixed with 4% paraformaldehyde for 10 min at 4°C and blocked with Block Ace (Yukijirushi, Sapporo, Japan) for 40 min at room temperature. Cells were incubated with mouse anti-FLAG monoclonal antibody (Sigma, St. Louis, Mo., USA) for 12 h at 4°C, followed by incubation with biotinylated horse anti-mouse IgG (Vector, Burlingame, Calif., USA) and fluorescein avidin D (Vector). Nuclei were made visible with propidium iodide (PI).

Electrophoretic mobility shift assay (EMSA) and western blot analysis. We used in vitro translated proteins or nuclear cell extract. Wild-type and mutant HNF-4 α proteins were synthesised using 1 μ g of pcDNA3.1 construct and TNT T7 Transcription/Translation System (Promega). We transfected HeLa cells (1×10^6) with 8 μ g of WT or mutant HNF-4 α and nuclear extracts were prepared as described [7]. We used 2 μ l of the in vitro translated proteins or 3 μ g of nuclear proteins for EMSA. Proteins were incubated with 32 P-labelled oligonu-

cleotide containing the HNF-4 α binding site of the human *HNF-1A* promoter (5'-GGCTGAAGTCCAAAGTT-CAGTCCCTTCGC-3') in a 20 μ l reaction mixture containing 20 mmol/l HEPES (pH 7.9), 75 mmol/l KCl, 3% Ficoll, 1 mmol/l MgCl₂, 0.1 mmol/l EDTA, 1 mmol/l dithiothreitol (DTT), 2 μ g poly (dI-dC) at 25°C for 30 min. The DNA-protein complexes were analysed on 5% polyacrylamide gels using 0.5 $\times$ TRIS-borate/EDTA before (TBE) buffer. The polyclonal antibody (α 445) [2] was obtained from Dr F.M. Sladek (University of California). The images were quantified using ScanningImager (Molecular Dynamics, Sunnyvale, Calif., USA). Western blot analysis was done using anti-HNF-4 α antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) [7].

Results

Subcellular localisation of wild-type and mutant HNF-4 α . We transfected HeLa cells with FLAG tagged WT-HNF-4 α and R127W-HNF-4 α constructs (Fig. 1a-d). Out of 133 cells 18 (13.5%) and out of 143 cells 17 (11.9%) were positive for staining, respectively. Immunostaining suggested that both WT-HNF-4 α and R127W-HNF-4 α were localised in the nucleus. The nuclear localisation of both proteins was confirmed by the nuclear staining with PI (Fig. 1c, d), indicating that this mutation does not affect the intracellular localisation.

DNA binding study. We carried out EMSA using in vitro translated HNF-4 α . The expressions of WT, R127W and CD1b-HNF-4 α translated in vitro were similar (Fig. 1e). The WT-HNF-4 α construct bound to the oligonucleotide and the binding was blocked by the addition of 50-fold excess of unlabelled probes (Fig. 1f, lanes 1 and 2). The DNA-protein complex was supershifted by the addition of the polyclonal anti-HNF-4 α antiserum, but normal serum did not affect the complex (lanes 3 and 4). The binding of R127W-HNF-4 α was decreased by 47.0% compared with that of WT-HNF-4 α (lanes 1 and 5).

Equal amounts of WT and CD1b or R127W and CD1b proteins were mixed and assayed for DNA binding. An additional protein/DNA complex was observed with an intermediate mobility (lanes 7 and 8). These results suggest that R127W-HNF-4 α forms a homodimer and heterodimer.

We also carried out EMSA using nuclear extracts from HNF-4 α transfected HeLa cells. We confirmed that the expression level of WT-HNF-4 α and R127W-HNF-4 α was the same by western blot analysis (Fig. 1g). We found WT-HNF-4 α specifically bound to the oligonucleotide as described above (Fig. 1h, lanes 1-4) but Q268X-HNF-4 α failed to bind to the oligonucleotide (Fig. 1h, lane 6) as noted previously [8]. The binding of R127W-HNF-4 α was decreased by 61.7% compared with that of WT-HNF-4 α (lanes 1 and 5). These data suggest that the R127W mutation affects the DNA binding ability of HNF-4 α .

Fig. 1. **a-d** Subcellular localisation of WT and R127W HNF-4 α in HeLa cells. HeLa cells were transfected with 0.5 μ g of FLAG-epitope tagged WT and mutant HNF-4 α constructs. Expression of HNF-4 α -FLAG protein (green fluorescence) (**a, b**). Double exposed with PI staining (red colour) showed yellow fluorescence in the nuclei (**c, d**). (**a, c**) WT-HNF-4 α , (**b, d**) R127W-HNF-4 α . Bar = 30 μ m (**a, b**). Bar = 15 μ m (**c, d**). **e** Expression of in vitro translated WT, R127W and CD1b-HNF-4 α proteins. HNF-4 α proteins were in vitro translated and separated on 10 % SDS-polyacrylamide gel. **f** EMSA with in vitro translated proteins. Proteins were incubated with 32 P-labelled oligonucleotide containing the HNF-4 α binding site of the human HNF-1 α promoter (5'-GGCTGAAGTCCAAAGTTCAGTCCCTTCGC-3') in a 20 μ l reaction mixture containing 20 mmol/l HEPES (pH 7.9), 75 mmol/l KCl, 3 % Ficoll, 1 mmol/l MgCl₂, 0.1 mmol/l EDTA, 1 mmol/l DTT, 2 μ g poly (dI-dC) at 25°C for 30 min. The DNA-protein complexes were analysed on 5 % polyacrylamide gels using 0.5 $\times$ TBE buffer. Lane 1: WT-HNF-4 α , lane 2: WT-HNF-4 α and competitor, lane 3: WT-HNF-4 α and anti-HNF-4 α antiserum, lane 4: WT-HNF-4 α and normal control serum, lane 5: R127W-HNF-4 α , lane 6: CD1b-HNF-4 α , lane 7: CD1b and WT-HNF-4 α , lane 8: CD1b and R127W-HNF-4 α . The arrow indicates the position of the heterodimer between CD1b and WT/R127W. **g** Western blot analysis of WT and R127W-HNF-4 α . Expression of WT and R127W-HNF-4 α in HeLa cells. **h** EMSA with nuclear proteins extracted from HNF-4 α transfected HeLa cells. Lane 1: WT-HNF-4 α , lane 2: WT-HNF-4 α and competitor, lane 3: WT-HNF-4 α and anti-HNF-4 α antiserum, lane 4: WT-HNF-4 α and normal control serum, lane 5: R127W-HNF-4 α , lane 6: Q268X-HNF-4 α

Transactivation study. The transactivation ability of WT and mutant HNF-4 α was studied in HeLa cells that lack endogenous HNF-4 α activity. We found WT-HNF-4 α stimulated the transcription of the *HNF-1 α* and *PKL* promoter-luciferase reporter constructs and generated a statistically significant increase in the reporter gene activity whereas Q268X-HNF-4 α did not (Fig. 2a, b). The transactivation activity of R127W-HNF-4 α was 70.3 % ($p = 0.0072$) (*HNF-1 α*) and 73.5 % ($p = 0.0045$) (*PKL*) of that of WT-HNF-4 α . A reporter construct with a heterologous *TK* promoter and HNF-4 α binding sequence as an enhancer (pHNF4-tk-Luc) was also used to confirm the impaired transactivation activity of R127W-HNF-4 α . The transactivation activity of R127W-HNF-4 α was decreased by 66.2 % ($p = 0.001$) compared with WT-HNF-4 α (Fig. 2c) suggesting that R127W-HNF-4 α represents a loss-of-function mutation.

The transactivation activity of R127W-HNF-4 α was further investigated using MIN6 cells, an insulin secreting cell line that has endogenous HNF-4 α activity. The transcription of *HNF-1 α* , *PKL* and heterologous *TK* reporters by R127W-HNF-4 α were reduced compared with WT-HNF-4 α : 77.7 % ($p = 0.033$), 77.8 % ($p = 0.0075$) and 48.2 % ($p = 0.002$), respectively (Fig. 2d, e and f).

Because R127W is located in a region of HNF-4 α that interacts with the coactivator CBP, this mutation could impair the functional interaction of HNF-4 α with CBP. Co-transfection of HNF-4 α and CBP constructs stimulated the pHNF4-tk-Luc reporter gene activity by 4.0-fold compared with the transfection of HNF-4 α alone. The CBP construct also increased the transcriptional activity of R127W-HNF-4 α by 4.7-fold (Fig. 2g), suggesting that there is no impaired synergy between R127W-HNF-4 α and CBP in the system.

Discussion

We have previously shown that mutations in the *MODY3/HNF-1 α* gene result in diverse functional consequences including impairment of DNA binding ability, improper intracellular localisation and reduced transcriptional activity [7]. In this study, we have shown that the nuclear import and functional synergy with CBP of R127W-HNF-4 α are normal and the DNA binding and the transactivation ability of this mutation are reduced. Impairment of the synergistic effect between another missense mutation (E276Q-HNF-4 α) and COUP-TFII on the transcription of HNF-1 α has been reported [9]. The decreased binding ability of R127W-HNF-4 α suggests the conserved arginine residue is important for the binding. The decreased binding ability of R127W-HNF-4 α might account for its reduced transactivation activity.

Fig. 2. **a-f** Transactivation activities of WT and mutant HNF-4 α in HeLa cells (**a, b, c**) and MIN6 cells (**d, e, f**). Cells were transfected with 0.5 μ g of the WT or mutant HNF-4 α constructs and 0.5 μ g of the reporter genes together with 10 ng of pRL-SV40. HNF-1 α reporter (**a, d**), PKL reporter (**b, e**), pHNF4-tk-Luc (**c, f**). The results were from three independent experiments; means $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$. **g** Synergistic effect between HNF-4 α and CBP on the pHNF4-tk-Luc reporter. HeLa cells were transfected with 0.1 μ g of pcDNA3.1-WT- or R127W-HNF-4 α and 1 μ g of pRc/RSV-CBP. The results were from three independent experiments; means $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$. **h** The pedigree of the family with the R127W mutation [3]. Subjects with diabetes are noted by filled symbols and non-diabetic (or not tested) by open symbols. The age at diagnosis and the genotype are noted: N, normal; M, mutant (R127W)

The R127W mutation was present in 3 out of 5 of the diabetic patients we examined (in total 4 because subject II-2 can be assumed to have the R127W mutation) [3] (Fig. 2h). The age at diagnosis of the diabetic patients with the mutation ranged from 17–90 years [3]. Such a variable penetrance of the mutant allele could be a consequence of the relatively mild nature of this mutation. There were two affected members who did not have the mutation (III-3 and III-6). They could have another form of Type II diabetes because diabetes was transmitted from both parents. Such aetiological heterogeneity has been noted previously [10].

It has recently been reported that the transactivation activity of R127W-HNF-4 α is normal and sug-

gested that this mutation is a rare polymorphism [5]. Our results suggest otherwise. We do not have an adequate explanation to account for the difference. That study used two reporter constructs; pZLHIV-A1-4 which contains four HNF4 binding sites of the apolipoprotein A1 upstream of an HIV-LTR basal promoter and pLZ100 which contains apolipoprotein CIII promoter containing one HNF4 binding site. It is known that HNF-4 α activity depends on the promoter context [6]. The difference of the reporter systems might have contributed to the different results.

The transcription of the *HNF-1 α* and *PKL* genes by R127W-HNF-4 α were decreased. The HNF-1 α transcription factor regulates the expression of genes involved in the glucose homeostasis (e.g. *GLUT2*, *PKL* and insulin) in pancreatic beta-cells [7]. Thus, the mechanism by which R127W-HNF-4 α leads to beta-cell dysfunction could be through HNF-1 α both directly and indirectly. Identification of the target genes of HNF-4 α action in the beta cell should lead to a better understanding of the molecular pathogenesis of MODY1/HNF-4 α diabetes.

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