



# In silico and in vitro analyses of the pathological relevance of the R258H mutation of hepatocyte nuclear factor 4 $\alpha$ identified in maturity-onset diabetes of the young type 1

Sugawara, Kenji ; Nomura, Kazuhiro ; Okada, Yuko ; Sugano, Aki ;  
Matsumoto, Masaaki ; Takarada, Toru ; Takeuchi, Atsuko ; Awano,...

---

(Citation)

Journal of Diabetes Investigation, 10(3):680-684

(Issue Date)

2019-05

(Resource Type)

journal article

(Version)

Version of Record

(Rights)

© 2018 The Authors. Journal of Diabetes Investigation published by Asian Association for the Study of Diabetes (AASD) and John Wiley & Sons Australia, Ltd.  
This is an open access article under the terms of the Creative Commons Attribution - NonCommercial License, which permits use, distribution and reproduction in any mediu...

(URL)

<https://hdl.handle.net/20.500.14094/90006079>



# ***In silico* and *in vitro* analyses of the pathological relevance of the R258H mutation of hepatocyte nuclear factor 4 $\alpha$ identified in maturity-onset diabetes of the young type 1**

Kenji Sugawara<sup>1</sup>, Kazuhiro Nomura<sup>1</sup>, Yuko Okada<sup>1</sup> , Aki Sugano<sup>2</sup>, Masaaki Matsumoto<sup>3</sup>, Toru Takarada<sup>4</sup>, Atsuko Takeuchi<sup>4</sup>, Hiroyuki Awano<sup>3</sup> , Yushi Hirota<sup>1</sup> , Hisahide Nishio<sup>5</sup>, Yutaka Takaoka<sup>2</sup>, Wataru Ogawa<sup>1\*</sup> 

<sup>1</sup>Division of Diabetes and Endocrinology, Department of Internal Medicine, <sup>2</sup>Division of Medical Informatics and Bioinformatics, <sup>3</sup>Department of Pediatrics, Kobe University Graduate School of Medicine, <sup>4</sup>Kobe Pharmaceutical University, and <sup>5</sup>Department of Community Medicine and Social Healthcare Science, Kobe University Graduate School of Medicine, Kobe, Japan

## **Keywords**

Hepatocyte nuclear factor 4 $\alpha$ , Insulin secretion, Maturity-onset diabetes of the young type 1

## **\*Correspondence**

Wataru Ogawa  
Tel.: +81-78-382-5861  
Fax: +81-78-382-2080  
E-mail address:  
ogawa@med.kobe-u.ac.jp

*J Diabetes Investig* 2019; 10: 680–684

doi: 10.1111/jdi.12960

## **ABSTRACT**

Mutations of the hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ) gene give rise to maturity-onset diabetes of the young type 1. Although many such mutations have been identified in affected individuals, part of these mutations has been characterized with regard to their pathological relevance. We here identified a missense mutation (c.773G>A, p.R258H) of *HNF4A* in a mother and daughter with early-onset diabetes and impaired insulin secretion. *In silico* simulation and *in vitro* luciferase reporter analyses showed that the mutation impairs the stability of self-dimerization and the transactivation activity of HNF4 $\alpha$ . Although arginine-258 does not appear to participate directly in dimerization, its mutation alters the electrostatic surface potential of the dimer interface. Our results thus suggest that this mutation impairs the function of HNF4 $\alpha$  and thereby contributes to the pathogenesis of maturity-onset diabetes of the young type 1.

## **INTRODUCTION**

Maturity-onset diabetes of the young (MODY) is a monogenic form of diabetes characterized by autosomal dominant inheritance and a defect in pancreatic  $\beta$ -cell function<sup>1</sup>. Mutations in the gene for the transcription factor hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ) give rise to MODY1, which accounts for ~5% of all MODY cases<sup>1,2</sup>. Although >100 mutations of *HNF4A* have been identified in individuals clinically diagnosed with MODY1, part of these mutations has been characterized with regard to their pathological relevance<sup>3</sup>.

Here, we report two cases of MODY1 in a mother and daughter with a missense mutation (c.773G>A, p.R258H) of *HNF4A*. Although this mutation has been previously identified in siblings diagnosed with MODY1<sup>4</sup>, functional analysis of the mutant protein has not been carried out. We therefore investigated the pathological relevance of this mutation with *in silico* simulation analysis and *in vitro* experiments.

## **METHODS**

### **Gene Sequencing**

All exons of *HNF4A* were amplified by polymerase chain reaction from genomic deoxyribonucleic acid (DNA) isolated from peripheral blood cells and with primers listed in Table S1. The polymerase chain reaction products were directly sequenced with the use of a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) and a 3730 DNA analyzer.

All the data of the proband and her mother presented in this study, including the results of genetic analyses, were collected for the diagnosis and the treatment of their disease. The proband and her mother provided written consent to publish their clinical data.

### ***In silico* simulation analysis**

The three-dimensional structure and electrostatic surface potential of mutant forms of HNF4 $\alpha$  were analyzed as described previously<sup>5</sup> with the use of three-dimensional structure information for wild-type human HNF4 $\alpha$  obtained from Protein Data Bank

Received 29 August 2018; revised 4 October 2018; accepted 15 October 2018

(PDB ID: 3FS1). The binding stability of dimerization was evaluated with the ZDOCK score calculated with ZDOCK software<sup>6</sup>, as described in Data S1.

### Luciferase Reporter Assay

The promoter region of the *ABCC6* gene (nucleotides -332 to +72 [relative to the transcription start site]), a target of HNF4 $\alpha$ <sup>7</sup>, was subcloned into the pGL3 Basic Vector (Promega, Madison, WI, USA) to yield pABCC6-luc. COS-7 cells were transfected with pABCC6-luc, pcDNA3.1 encoding wild-type or mutant forms of HNF4 $\alpha$ 2 (or empty pcDNA3.1) and a plasmid encoding  $\beta$ -galactosidase. Luciferase and  $\beta$ -galactosidase activities in cell lysates were measured 48 h after transfection, and the former was normalized by the latter.

## RESULTS

### Case

The proband, a Japanese woman born with birthweight of 2,554 g at 36 weeks-of-gestation was diagnosed with diabetes at 10 years of age, and was admitted to Kobe University Hospital, Kobe, Japan, for evaluation and management of her disease at age 16 years. She was treated with basal-bolus insulin therapy. On admission, her hemoglobin A<sub>1c</sub> level was 14.5%, and antibodies to glutamic acid decarboxylase and to insulinoma-associated protein-2 were negative (Table S2). She had no severe hypoglycemic episode and apparent diabetic microvascular or macrovascular complications. Her mother was the only family member also known to have diabetes (Figure S1), with which she had been diagnosed at age 14 years.

Insulin secretion was evaluated in the proband after her glycemia had been lowered sufficiently. The average of the sum of serum C-peptide immunoreactivity ( $\Sigma$ CPR) during an oral glucose tolerance test (8.6 ng/mL) was markedly lower than that previously reported for individuals with diabetes (~20 ng/mL; Table 1)<sup>8</sup>. The secretion of C-peptide (peak CPR 2.77 ng/mL), but not that of glucagon, in response to an arginine challenge test was lower than that described for type 2 diabetes patients (peak CPR ~4 ng/mL; Table 1)<sup>9</sup>. The secretion of C-peptide during a glucagon challenge test ( $\Delta$ CPR 1.2 ng/mL) was also lower than that previously reported for type 2 diabetes patients (average  $\Delta$ CPR 2.0 ng/mL; Table 1)<sup>10</sup>. A glucagon challenge test carried out with the proband's mother showed that the C-peptide response was even lower than that of the proband (Table 1). These results were indicative of impaired  $\beta$ -cell function in both the proband and her mother. Concomitantly, insulin therapy was considered to be necessary for this patient.

### Mutation Analysis of HNF4A

Mutational screening of genes known to be responsible for MODY showed that both the proband and her mother harbored a heterozygous mutation (c.773G>A) of *HNF4A* that alters the encoded amino acid sequence (p.R258H; Figure S2). HNF4 $\alpha$  forms a homodimer through its ligand-binding domain that is essential for its transactivation activity<sup>11</sup>. The crystal

**Table 1** | Evaluation of insulin secretion in the proband and her mother

|                             | Proband | Mother |
|-----------------------------|---------|--------|
| Oral glucose tolerance test |         |        |
| C-peptide (ng/mL)           |         |        |
| Pre                         | 0.85    |        |
| 30 min                      | 2.53    |        |
| 60 min                      | 2.77    |        |
| 120 min                     | 1.79    |        |
| Glucose (mg/dL)             |         |        |
| Pre                         | 121     |        |
| 30 min                      | 199     |        |
| 60 min                      | 321     |        |
| 120 min                     | 323     |        |
| $\Sigma$ CPR (ng/mL)        | 8.6     |        |
| Arginine challenge test     |         |        |
| C-peptide (ng/mL)           |         |        |
| Pre                         | 0.85    |        |
| 30 min                      | 2.53    |        |
| 60 min                      | 2.77    |        |
| 120 min                     | 1.79    |        |
| Glucagon (pg/mL)            |         |        |
| Pre                         | 113     |        |
| 30 min                      | 521     |        |
| 60 min                      | 365     |        |
| 120 min                     | 134     |        |
| Glucagon challenge test     |         |        |
| Glucose (mg/dL)             |         |        |
| Pre                         | 98      | 94     |
| Post                        | 131     | 105    |
| C-peptide (ng/mL)           |         |        |
| Pre                         | 1.0     | 1.8    |
| Post                        | 2.2     | 2.6    |

Plasma glucose or glucagon as well as serum C-peptide concentrations before and 30, 60 or 120 min after the oral administration of glucose (75 g) or intravenous administration of arginine (0.5 g/kg) were measured. Plasma glucagon was measured with a radioimmunoassay (BML, Tokyo, Japan). For the glucagon challenge test, glucagon (1 mg) was injected intravenously after the participants had been deprived of food overnight, with plasma glucose and serum C-peptide being measured before (pre) and 6 min after (post) the injection.  $\Sigma$ CPR, sum of serum C-peptide immunoreactivity.

structure of a complex of HNF4 $\alpha$  with DNA and a coactivator<sup>11</sup> shows that Arg<sup>258</sup> (R258) is located near the dimer interface (Figure S3).

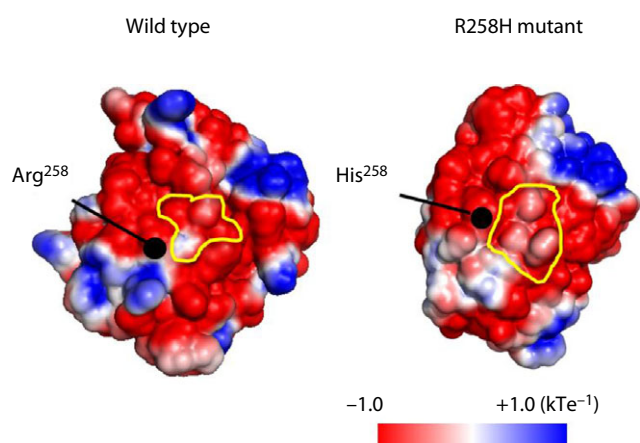
### In Silico Simulation Analysis

With the use of *in silico* simulation, we constructed the three-dimensional structures of dimers formed by wild-type HNF4 $\alpha$  or by MODY1-associated mutants (E276Q, R258H, V255M) that harbor amino acid substitutions near the dimer interface, and we calculated the ZDOCK score as a measure of dimer stability. The transactivation activity of the E276Q mutant was previously shown to be impaired *in vitro*<sup>12</sup>. The ZDOCK scores of heterodimers formed by the E276Q or R258H mutants with

**Table 2** | *In silico* simulation analysis of hepatocyte nuclear factor 4 $\alpha$  dimer stability

|             | ZDOCK score        | Obtained dimers | <i>P</i> -value 1 | <i>P</i> -value 2 |
|-------------|--------------------|-----------------|-------------------|-------------------|
| Wild type   |                    |                 |                   |                   |
| Homodimer   | 133.31 $\pm$ 20.35 | 24              | —                 | —                 |
| R258H       |                    |                 |                   |                   |
| Heterodimer | 95.64 $\pm$ 7.66   | 15              | <0.001            | —                 |
| Homodimer   | 81.90 $\pm$ 9.48   | 35              | <0.001            | <0.001            |
| V255M       |                    |                 |                   |                   |
| Heterodimer | 120.64 $\pm$ 33.66 | 47              | NS                | —                 |
| Homodimer   | 108.35 $\pm$ 33.55 | 62              | <0.05             | NS                |
| E276Q       |                    |                 |                   |                   |
| Heterodimer | 113.25 $\pm$ 33.09 | 25              | <0.01             | —                 |
| Homodimer   | 93.27 $\pm$ 16.80  | 54              | <0.001            | <0.01             |

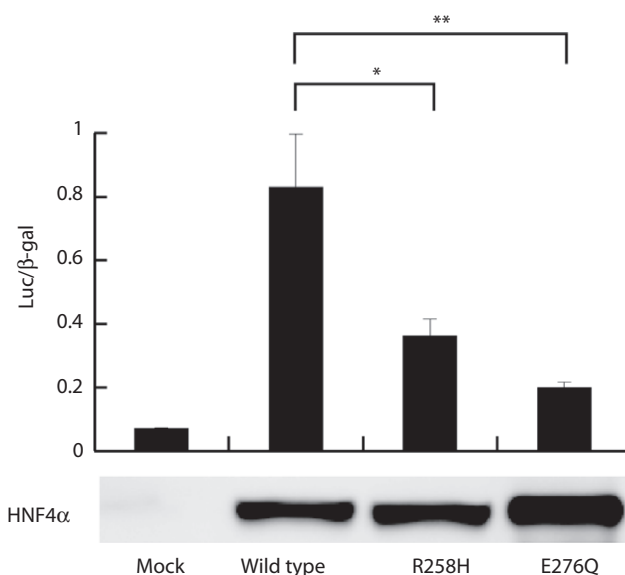
ZDOCK scores are mean  $\pm$  standard deviation. Obtained dimers derived from 2,000 results of docking analyses were chosen on the basis of similar structure of mouse hepatocyte nuclear factor 4 $\alpha$  docking. *P*-values 1 and 2 are for comparison with the wild-type homodimer or with the corresponding heterodimer formed with the wild-type protein. NS, not significant ( $P \geq 0.05$ ).



**Figure 1** | Electrostatic surface potential of wild-type and R258H mutant forms of hepatocyte nuclear factor 4 $\alpha$ . Potentials from negative to positive are shown as red to blue, respectively. Dimer interfaces are outlined in yellow. [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

the wild-type protein were lower than that of the wild-type homodimer, and the scores of the mutant homodimers were lower than those of the corresponding heterodimers, suggestive of a pathological role for these mutations (Table 2). In contrast, the ZDOCK score of the heterodimer formed by the wild-type protein and the V255M mutant, which manifests normal transactivation activity<sup>12</sup>, was similar to that of the wild-type homodimer, suggesting that this mutation is of little pathogenic relevance (Table 2).

We also analyzed the electrostatic surface potential of the dimer interface for wild type and R258H. Although the electrostatic potential at position 258 is similar, the surface of helix 10/11 (H10/11), which plays an important role in dimerization<sup>11</sup>, is acidic in the wild type, but neutral in the R258H



**Figure 2** | Luciferase reporter assay of transactivation activity for wild-type and mutant forms of hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ). The luciferase/ $\beta$ -galactosidase (Luc/ $\beta$ -gal) activity ratios are shown as mean  $\pm$  standard error of the mean from three independent experiments. \* $P < 0.05$  (Student's *t*-test). The cell lysates were subjected to immunoblot analysis with anti-HNF4 $\alpha$  antibody (sc-374229; Santa Cruz Biotechnology, Santa Cruz, CA, USA), \*\* $P < 0.01$

(Figure 1). Collectively, these results suggest that the R258H mutation alters the function of HNF4 $\alpha$  by affecting protein structure adjacent to its position.

#### Luciferase Reporter Analysis

Finally, we measured the transactivation activity of the R258H mutant *in vitro*. Forced expression of wild-type HNF4 $\alpha$  in COS-7 cells resulted in a marked increase in luciferase

expression driven by the *ABCC6* promoter (Figure 2). The luciferase activity in cells expressing the R258H or E276Q mutants was significantly lower than that in those expressing the wild-type protein (Figure 2), providing further support for the pathological relevance of the R258H mutation.

## DISCUSSION

We have characterized the pathological relevance of a mutation of *HNF4A* identified in a mother and daughter with early-onset diabetes and impaired insulin secretion. Both women were thus diagnosed with MODY1. Although the mutation (c.773G>A) was previously identified in a single family<sup>4</sup>, we now show that it confers a functional defect with the use of *in silico* simulation analysis and *in vitro* experiments.

The simulation analysis and luciferase reporter assay showed that the stability of the dimers formed by and the transactivation activity of the R258H mutant are impaired. The crystal structure of HNF4 $\alpha$  shows that Arg<sup>258</sup> is located near the dimer interface<sup>11</sup>. Whereas Arg<sup>258</sup> does not directly participate in dimerization, it forms an intramolecular salt-bridge with Glu<sup>262</sup>, as well as hydrogen bonds with Ser<sup>337</sup> and Gln<sup>341</sup> (Figure S3). These hydrophilic interactions might stabilize the orientation of H10/11, which plays a pivotal role in dimerization. The electrostatic surface potential of H10/11 differs between the wild-type and R258H mutant proteins. Instability of dimerization conferred by the R258H mutation might thus result from an allosteric structural change at H10/11.

## ACKNOWLEDGMENTS

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan to KS (16K19537) and YH (16K09750).

## DISCLOSURE

The authors declare no conflict of interest.

## REFERENCES

1. Horikawa Y. Maturity-onset diabetes of the young as a model for elucidating the multifactorial origin of type 2 diabetes mellitus. *J Diabetes Investig* 2018; 9: 704–712.
2. Yamagata K, Furuta H, Oda N, *et al.* Mutations in the hepatocyte nuclear factor-4 $\alpha$  gene in maturity-onset diabetes of the young (MODY1). *Nature* 1996; 384: 458–460.
3. Colclough K, Bellanne-Chantelot C, Saint-Martin C, *et al.* Mutations in the genes encoding the transcription factors hepatocyte nuclear factor 1 $\alpha$  and 4 $\alpha$  in maturity-onset diabetes of the young and hyperinsulinemic hypoglycemia. *Hum Mutat* 2013; 34: 669–685.
4. Motzkau M, Meyer P, Mertens PR, *et al.* Monogenic diabetes in a family with 2 unknown HNF-4A gene mutations. *Exp Clin Endocrinol Diabetes* 2012; 120: 89–90.
5. Nakano E, Ono R, Masaki T, *et al.* Differences in clinical phenotype among patients with XP complementation group D: 3D structure and ATP-docking of XPD *in silico*. *J Invest Dermatol* 2014; 134: 1775–1778.
6. Chen R, Li L, Weng Z. ZDOCK: an initial-stage protein-docking algorithm. *Proteins* 2003; 52: 80–87.
7. de Boussac H, Ratajewski M, Sachrajda I, *et al.* The ERK1/2-hepatocyte nuclear factor 4 $\alpha$  axis regulates human *ABCC6* gene expression in hepatocytes. *J Biol Chem* 2010; 285: 22800–22808.
8. Maruyama T, Tanaka S, Shimada A, *et al.* Insulin intervention in slowly progressive insulin-dependent (type 1) diabetes mellitus. *J Clin Endocrinol Metab* 2008; 93: 2115–2121.
9. Tsuchiyama N, Takamura T, Ando H, *et al.* Possible role of alpha-cell insulin resistance in exaggerated glucagon responses to arginine in type 2 diabetes. *Diabetes Care* 2007; 30: 2583–2587.
10. Fujioka Y, Okura T, Sumi K, *et al.* Normal meal tolerance test is preferable to the glucagon stimulation test in patients with type 2 diabetes that are not in a hyperglycemic state: comparison with the change of C-peptide immunoreactivity. *J Diabetes Investig* 2018; 9: 274–278.
11. Chandra V, Huang P, Potluri N, *et al.* Multidomain integration in the structure of the HNF-4 $\alpha$  nuclear receptor complex. *Nature* 2013; 495: 394–398.
12. Navas MA, Munoz-Elias EJ, Kim J, *et al.* Functional characterization of the MODY1 gene mutations HNF4 (R127W), HNF4(V255M), and HNF4(E276Q). *Diabetes* 1999; 48: 1459–1465.

## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** | Pedigree of the proband. Males are shown as squares and females as circles, the proband is indicated by the arrow, and family members known to have diabetes are shaded black. Ages at the time of the present study are indicated in years (y).

**Figure S2** | Sequence analysis of hepatocyte nuclear factor 4 $\alpha$  (HNF4A). (a) Sequences of the HNF4A gene of the proband and her mother. Partial sequences surrounding the mutation site (arrow) of the proband and her mother, as well as the corresponding sequence for a control individual are shown. (b) Domain structure of HNF4 $\alpha$  and partial sequence surrounding the mutation site are shown. The substitution of amino acid is shown in red.

**Figure S3** | Mapping of Arg258 to the crystal structure of human hepatocyte nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ). The structure of full-length HNF4 $\alpha$  complexed with deoxyribonucleic acid and a coactivator (PDB ID: 4IQR) is presented. The left panel shows the

entire complex, whereas the right panel shows a zoomed-in image of the dimer interface. Three residues, Glu 262, Ser337 and Gln341, involved in the interaction with Arg258 are shown.

**Table S1** | Polymerase chain reaction primers used for amplification of hepatocyte nuclear factor 4 $\alpha$  exons.

**Table S2** | Laboratory data for the proband.

**Data S1** | Supporting method.