CLINICAL REPORT

Analysis of induced pluripotent stem cell clones derived from a patient with mosaic neurofibromatosis type 2

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Abstract

The diagnosis of mosaicism is challenging in patients with NF2 subset due to low variant allele frequency. In this study, we generated induced pluripotent stem cells (iPSCs) were generated from a patient clinically diagnosed with NF2 based on multiple schwannomas, including bilateral vestibular schwannomas and meningiomas. Genetic analysis of the patient's mononuclear cells (MNCs) from peripheral blood failed to detect *NF2* alteration but successfully found p.Q65X (c.193C > T) mutation in all separate tumors with three intracranial meningiomas and one intraorbital schwannoma, and confirming mosaicism diagnosis in *NF2* alteration using deep sequencing. Five different clones with patient-derived iPSCs were established from MNCs in peripheral blood, which showed sufficient expression of pluripotent markers. Genetic analysis showed that one of five generated iPSC lines from MNCs had the same p.Q65X mutation as that found in *NF2*. There was no significant difference in the expression of genes related to *NF2* between iPSC clones with the wild-type and mutant *NF2*. In this case, clonal expansion of mononuclear bone marrow-derived stem cells recapitulated mosaicism's genetic alteration in NF2. Patient-derived iPSCs from mosaic NF2 would contribute to further functional research of *NF2* alteration.

Keywords: neurofibromatosis type 2, mosaicism, induced pluripotent stem cells

INTRODUCTION

Neurofibromatosis type 2 (NF2) is an autosomal-dominant disease caused by germline alterations in the *NF2* tumor-suppressor gene. Patients with NF2 develop multiple intracranial and spinal tumors, including schwannomas and meningiomas (Asthagiri *et al*., 2009). The major forms of *NF2* alterations are nonsense, frameshift, splice-site, and missense mutations as well as large exon and in-frame deletions (Asthagiri *et al*., 2009; Evans *et al.*, 2020; Evans *et al.*, 2007; Moyhuddin *et al*., 2003; Teranishi *et al*., 2020). These genetic alterations in *NF2* are detected in peripheral blood samples of most patients with familial NF2, while many sporadic patients present with mosaicism of an *NF2* alteration (Evans *et al*., 2007; Halliday *et al*., 2017; Moyhuddin *et al*., 2003; Teranishi *et al.*, 2020). Mosaicism is generally diagnosed based on low variant allele frequency (VAF) in blood DNA or due to the presence of identical mutations in separate tumors (Moyhuddin *et al*., 2003). However, it is sometimes challenging to diagnose mosaicism in patients with NF2 due to low VAF in the bone marrow derived peripheral mononuclear cells (Evans *et al*., 2020; Teranishi *et al*., 2020).

Recently, induced pluripotent stem cells (iPSCs), established by somatic reprogramming using Yamanaka factors (Oct3/4, Sox2, KLF4, and c-Myc) (Takahashi *et al.*, 2007), have been applied to analyze various genetic disorders (Soga *et al*., 2015). In this report, we present the case of a woman with NF2, in whom mutation in bone marrow derived peripheral mononuclear cells was not detected by Sanger sequencing, but the mosaicism of *NF2* alteration in peripheral blood mononuclear cells was identified in the one of five clones of patient-derived iPSCs.

METHODS

Editorial Policies and Ethical Considerations

Approval was obtained from the Institutional Review Board (approval number: 015-0101). Written informed consent was obtained from the patient.

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A woman, who presented with bilateral hearing loss and was detected multiple intracranial schwannomas, including bilateral vestibular schwannomas, and meningiomas on radiological examinations, was diagnosed with NF2 based on the Manchester criteria (Evans *et al*., 1992; Smith *et al.*, 2017) when she was 33 years. The patient underwent resection surgery for anatomically distinct intracranial meningiomas in the left parietal convex, right parasagittal, and right falx at ages 37, 39, and 43 years, respectively. She also underwent resection surgery for intraorbital schwannoma at age of 43 years. We generated iPSCs from mononuclear cells (MNCs) present in the patient's peripheral blood using a Sendai virus (SeV) vector as previously described (Soga *et al*., 2015).

RESULTS

Patient-derived iPSCs were generated from peripheral blood MNCs, and the induction efficacy was 1.55%. We collected five clones of patient-derived iPSCs. All five clones with patient-derived iPSCs showed sufficient expression of pluripotent markers, and the disappearance of SeV was verified through nested reverse transcriptase-polymerase chain reaction (RT–PCR), fluorescent immunohistochemistry, and alkaline phosphatase staining, showing sufficient pluripotency in iPSCs derived from the patient with NF2 (Figure 1).

Genetic analysis of MNCs with DNA sequencing (Supplemental Methods, Supplemental Table) did not exhibit any pathogenic mutations in 17 exons and *NF2* splice sites. However, p.Q65X (c.193C > T) nonsense mutation in one *NF2* allele in separate tumors with three intracranial meningiomas and one schwannoma was detected [Figure 2(a)]. Although the

imperceptible mutant signal was observed on the Sanger sequencing of MNC, it was challenging to distinguish from noise [Figure 2(a)]. Deep sequencing with amplicon sequencing (Supplemental Methods) for MNC was conducted and its VAF in this allele was identified as 7.92% [Figure 2(b)]. For the first sample of meningioma (meningioma 1), targeted amplicon sequencing of *NF2* was performed as previously described (Yuzawa *et al*., 2016), and the loss of *NF2* was identified [Figure 2(c)]. Because complete loss of *NF2* is frequent in meningiomas but uncommon as a germline mutation of NF2 (Moyhuddin *et al*., 2003; Teranishi *et al*., 2020; Yuzawa *et al*., 2016), NF2 p.Q65X nonsense mutation was considered an initial post-zygotic somatic mutation. Based on the peripheral blood genetic analysis, the patient was genetically diagnosed as a mosaic patient. Among the five clones of iPSCs, one iPSC line from MNCs had the same heterozygous mutation as that in *NF2* [Figure 2(d)]. Thus, clonal expansion of mononuclear bone marrow-derived stem cells using somatic reprogramming recapitulated mosaicism of genetic alteration in the patient with *NF2*.

Because merlin, a protein coded by *NF2*, suppresses various signaling pathways, including RhoGTPase family signaling, PI3K/mTOR/AKT signaling, and mammalian Hippo signaling (Petrilli & Fernandez-Valle, 2016), quantitative RT–PCR was conducted (Supplemental Methods) to assess the expression of genes in these pathways. However, there was no evidence of different expressions of these genes in the iPSC with *NF2* mutation compared to iPSC with wild-type *NF2* [Figure 2(e)].

DISCUSSION

The *NF2* genetic analysis methods commonly consisted of DNA sequencing in all 17 exons and multiple ligation-dependent probe amplification (Evans *et al*., 2020; Evans *et al*., 2007). Although *NF2* mutation is undetected in the blood, the same mutation in two separate tumors

indicates a mosaic (Evans *et al*., 2007). The combination of DNA sequencing and multiplex ligation-dependent probe amplification for blood and separate tumors has revealed mosaicism in 25%–33% of patients with NF2 (Evans *et al*., 2020; Moyhuddin *et al*., 2003). Recent studies have reported higher sensitivity and proportions of predicted mosaicism with 37.7%–59.7% using next-generation sequencing to detect *NF2* alteration with low VAF in patients with mosaic NF2 (Evans *et al*., 2020; Teranishi *et al*., 2020). Mosaicism in patients with NF2 has been speculated based on the VAF in the blood DNA or by identical mutations in different tumors in previous reports (Evans *et al*., 2020; Moyhuddin *et al*., 2003; Teranishi *et al*., 2020). A previous report has indicated that 25% with VAF is detectable using conventional Sanger sequencing, but 6.25% with VAF was quite a low signal of the mutant allele (Paganini *et al*., 2014), and 7.92%, in this case, was consistent with their report. Consistent with our result, patient-derived iPSC induction has been applied to discover mosaicism in other diseases, such as facioscapulohumeral dystrophy type-1, Klinefelter syndrome, Dravet syndrome, and neonatal-onset multisystem inflammatory disease (Fiacco, Alowaysi, Astro, & Adamo, 2020; Kawasaki *et al*., 2017; Maeda *et al*., 2016; van der Wal *et al*., 2019).

Mosaicism detection in NF2 using somatic reprogramming can manufacture isogenic control for *NF2* functional research since these clones have the same genetic background, except for *NF2* alteration. Our gene expression analysis exhibited no significant difference in expression of merlin-associated pathways. It was consistent with the fact that *NF2* is a tumor suppressor gene, and loss of heterogeneity is needed to cause aberrant activation or suppression in merlin-associated pathways. In this study, we firstly proved that monoallelic inactivating alteration in *NF2* does not affect to merlin-associated pathways using patient-derived iPSCs. However, because our analysis was conducted only in iPSC, expression of these genes in somatic cells is still unclear. Further analysis with differentiation induction for different kinds

of somatic cells or models with induction of biallelic inactivation of *NF2* would be needed. In addition, iPSCs derived from patient with mosaic NF2, including isogenic control with wild-type *NF2*, would contribute to discover novel functions of merlin.

There are some limitations in this study. Firstly, Low VAF of *NF2* is a significant concern in diagnosing mosaicism. Although *NF2* alterations were found in one of five iPSC clones, in this case, more clones would have been required to increase the diagnostic accuracy considering that 7.92% with VAF was identified using deep sequencing in this case. Current conventional methods hardly detect mosaicism since some patients harbor significantly low VAF with <1% germline *NF2* alteration (Teranishi *et al*., 2020). The availability of iPSCs for mosaic diagnosis is limited because the frequency of mutant iPSC clones depends on the VAF in the germline *NF2,* and the diagnosis of mosaic would not be achieved if iPSC clones do not possess *NF2* alteration. Furthermore, although blood samples can be used in the diagnosis of majority of mosaic NF-2 cases, which could not be diagnosed from blood but from the buccal mucosa or hair follicle was reported (Teranishi *et al*., 2020). No NF2 alteration would be detected from iPSC obtained from MNCs for such cases. Secondly, it is not sure whether the mutation frequency among iPSC clones completely reflects VAF in the germline. Because the culture condition for iPSC is different from the original environment of MNCs, a selective advantage might change the fraction of mutant iPSCs. Although data on gene expression analysis did not give the activation of merlin-associated pathways in iPSC harboring heterozygous *NF2* mutation and haploinsufficiency in NF-2 were reported only in peripheral nerves with neuropathy (Hanemann, Diebold, & Kaufmann, 2007), and not in other tissues, iPSC with heterozygous *NF2* alteration might have a selective advantage in culture conditions. Third, the cost and effort for iPSC generation cannot be ignored. Thus, iPSC establishment would be efficient for only a limited number of cases with NF2 to

diagnose mosaicism. However, as iPSC generation is increasingly common for regenerative medicine and disease research (Hanatani & Takasu, 2020; Yamanaka, 2020), it was considered essential to recognize that the technique of iPSC generation would recapitulate mosaicism.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

Author Contributions

Yukitomo Ishi performed the iPSC generation, molecular analysis and draft writing. Takumi Era performed the iPSC generation and reviewed the manuscript. Sayaka Yuzawa, Michinari Okamoto and Ryosuke Sawaya performed the molecular analysis. Hiroaki Motegi and Shunsuke Terasaka contributed to the acquisition of clinical data and samples. Shigeru Yamaguchi contributed to data acquisition and reviewed the manuscript. Kiyohiro Houkin and Miki Fujimura supervised the study. All authors approved the final version of the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon request.

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Figure Legends

Figure 1

Induced pluripotent stem cells (iPSCs) derived from the patient with neurofibromatosis type 2 and the genetic analysis results

(a) Bright-field image presenting a colony of patient-derived iPSC

(b) Nested reverse transcriptase-polymerase chain reaction exhibiting a positive expression of pluripotent markers and the absence of Sendai virus (SeV) contamination in five clones of the patient-derived iPSCs (PC1, positive control with human iPSC [201B7]; PC2, positive control with human iPSC with SeV contamination; NC, negative control with distilled water)

(c) Fluorescent immunohistochemical and alkaline phosphatase staining showing a positive expression of pluripotent markers

Figure 2

Genetic analysis of *NF2* gene in mononuclear cells (MNCs), tumors, and patient-derived iPSC clones

(a) Sanger sequencing showing a heterozygous p.Q65X mutation (black arrows) in exon-2 of *NF2* in the tumor specimen of three meningiomas and one schwannoma in separate locations (white arrows). The signal with this mutation was perceptible in mononuclear cells (MNCs), which was challenging to distinguish from noise.

(b) Read count of amplicon sequencing for *NF2* exon 2 in MNCs presenting variant allele frequency of mutant T-allele in *NF2* c.193 with 7.92%.

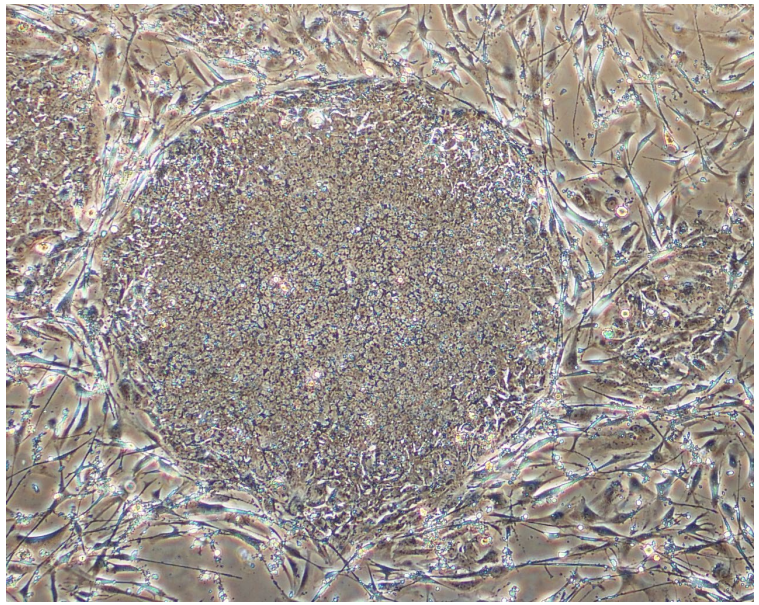
(c) Targeted amplicon sequencing of *ERBB2* in chromosome 17 (upper, 37855748−37924426) and *NF2* on chromosome 22 (lower, 29999919−30090863) presenting the whole deletion of *NF2*. The estimated copy number of *NF2* was 1.4–1.6. A score Q of *NF2* above 79 shows strong evidence for *NF2* loss; The score Q was 112 in this case.

(d) Sanger sequencing exhibiting a heterozygous p.Q65X mutation with a normal allele in exon 2 of *NF2* in iPSC clone #5 (arrow), but not in the other four clones

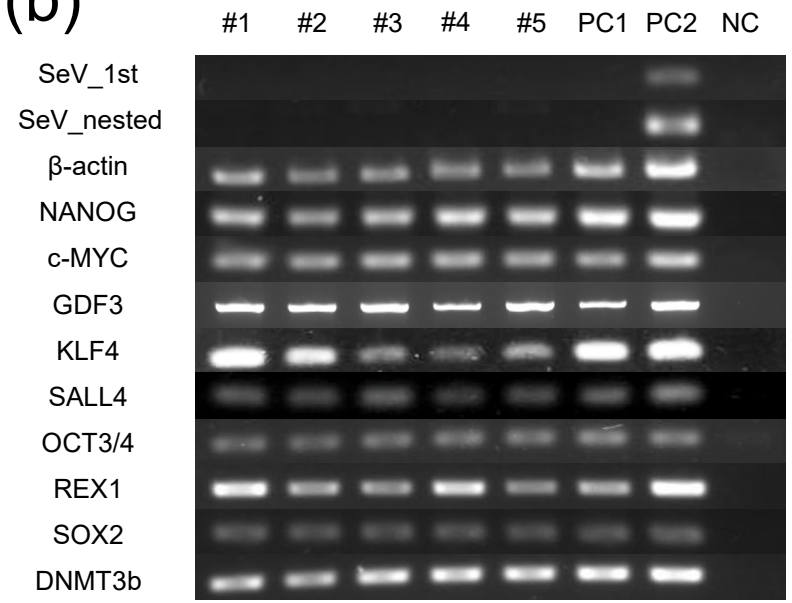
(e) Quantitative RT–PCR for genes of merlin-associated pathways in iPSC with wild-type *NF2* (#1–4) and mutant *NF2* (#5). Upper: Relative mRNA expression of *Rac1* and *PAK1* in RhoGTPase family signaling. Middle: Relative mRNA expression of *RICTOR* and *RAPTOR* in PI3K/mTOR/AKT signaling. Lower: Relative mRNA expression of *MST1*

and *YAP1* in Hippo signaling. No significant difference in mRNA expression between *NF2*-wild (#1–4) and *NF2*-mutant (#5) was observed in all these genes.

(a)



(b)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#2
#3
#4
#5
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PC2
NC
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SeV_nested
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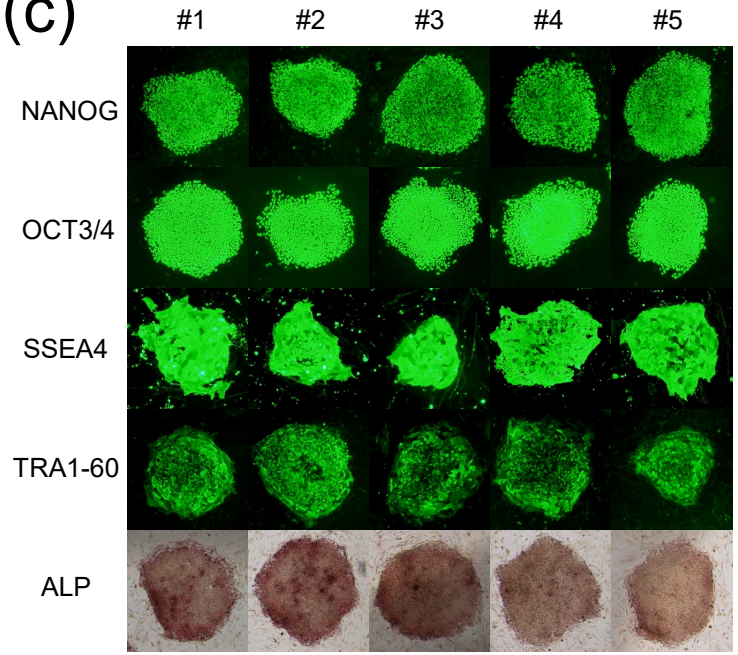
KLF4
SALL4
OCT3/4
REX1
SOX2
DNMT3b

(c)

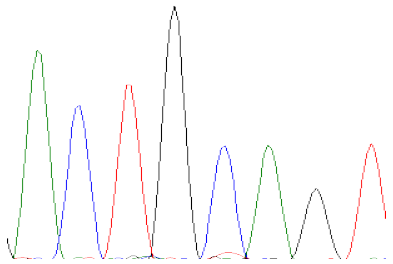

#1
#2
#3
#4
#5
NANOG
OCT3/4
SSEA4
TRA1-60
ALP

(a)

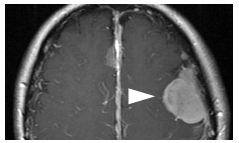
NF2 exon2

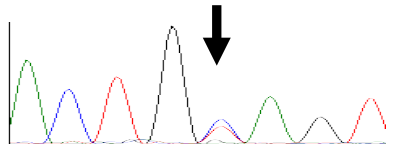
A C T G C A G T

Germline (MNC)



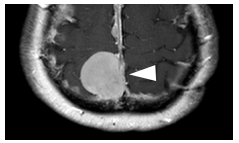
Meningioma 1

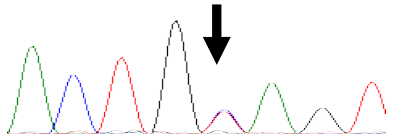




c.193C>T (p.Q65X)

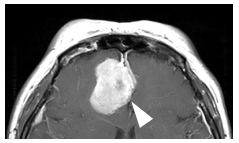
Meningioma 2

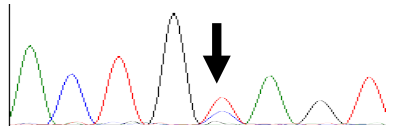




c.193C>T (p.Q65X)

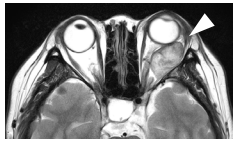
Meningioma 3

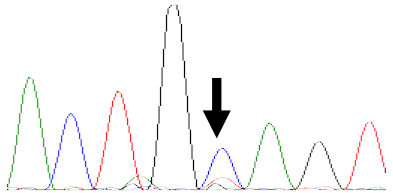




c.193C>T (p.Q65X)

Schwannoma





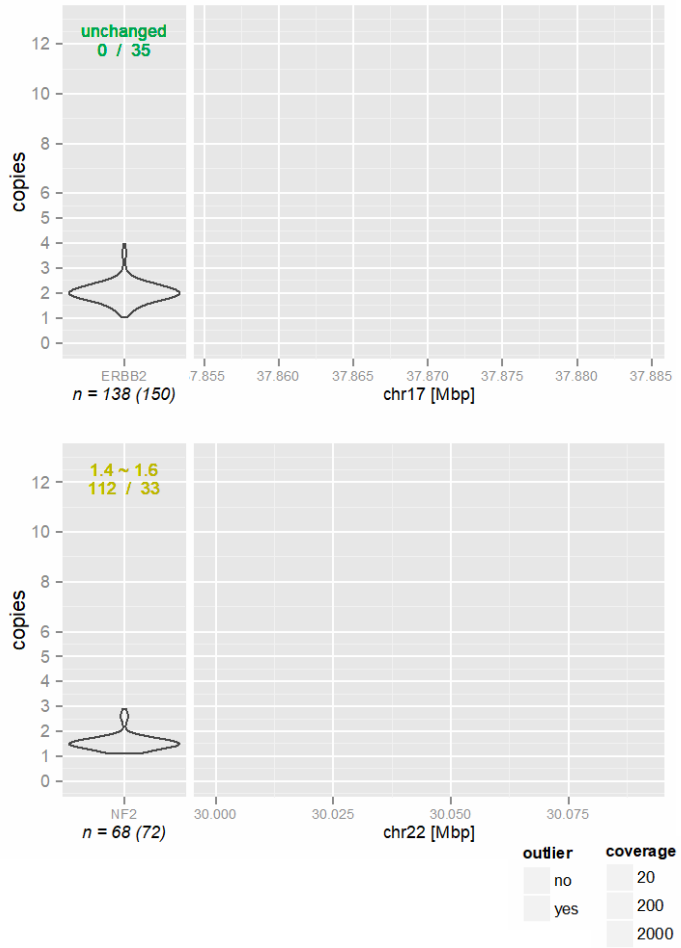
c.193C>T (p.Q65X)

(b)

Allele	Reads	%
C	99685	92.05
T	8583	7.92
A	21	0.019
G	2	0.0018

(c)

Meningioma 1

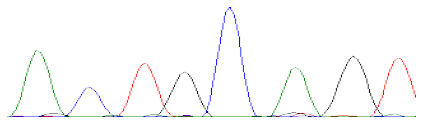


(d)

NF2 exon2

A C T G C A G T

iPSC #1



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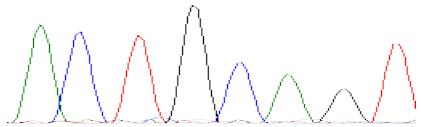

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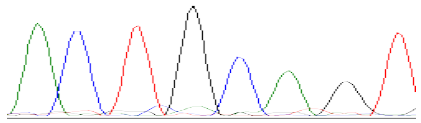
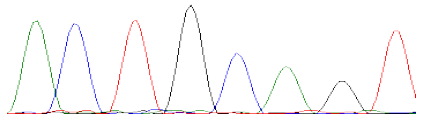
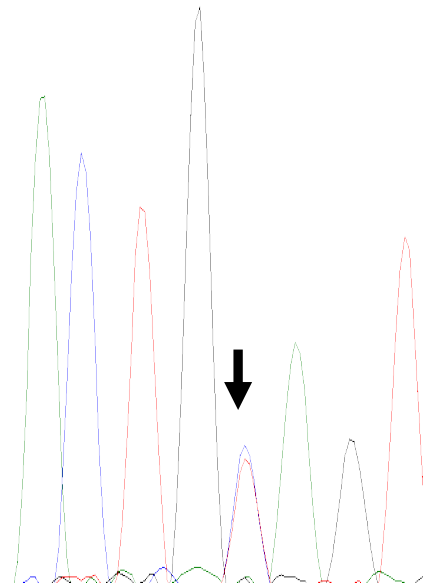


iPSC #4



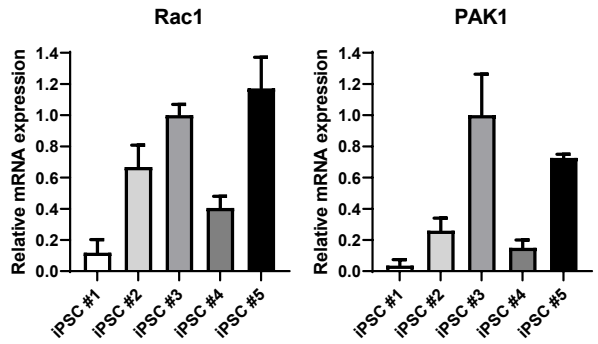
iPSC #5



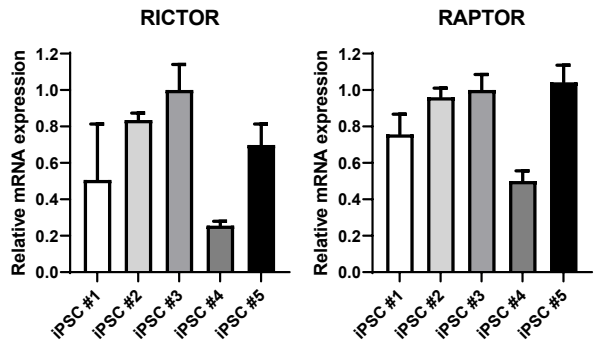
c.193C>T (p.Q65X)

(e)

RhoGTPase family signaling



PI3K/mTOR/AKT signaling



Hippo signaling