

HOKKAIDO UNIVERSITY

Title	Analysis of induced pluripotent stem cell clones derived from a patient with mosaic neurofibromatosis type 2
Author(s)	Ishi, Yukitomo; Era, Takumi; Yuzawa, Sayaka; Okamoto, Michinari; Sawaya, Ryosuke; Motegi, Hiroaki; Yamaguchi, Shigeru; Terasaka, Shunsuke; Houkin, Kiyohiro; Fujimura, Miki
Citation	American journal of medical genetics part A, 188(6), 1863-1867 https://doi.org/10.1002/ajmg.a.62700
Issue Date	2022-06
Doc URL	http://hdl.handle.net/2115/89793
Rights	This is the peer reviewed version of the following article: Ishi, Y., Era, T., Yuzawa, S., Okamoto, M., Sawaya, R., Motegi, H., Yamaguchi, S., Terasaka, S., Houkin, K., & Fujimura, M. (2022). Analysis of induced pluripotent stem cell clones derived from a patient with mosaic neurofibromatosis type 2. American Journal of Medical Genetics Part A, 188A: 1863–1867. which has been published in final form at 10.1002/ajmg.a.62700. This article may be used for non- commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions. This article may not be enhanced, enriched or otherwise transformed into a derivative work, without express permission from Wiley or by statutory rights under applicable legislation. Copyright notices must not be removed, obscured or modified. The article must be linked to Wiley 's version of record on Wiley Online Library and any embedding, framing or otherwise making available the article or pages thereof by third parties from platforms, services and websites other than Wiley Online Library must be prohibited.
Туре	article (author version)
Additional Information	There are other files related to this item in HUSCAP. Check the above URL.
File Information	AJMG 188a 1863-1867.pdf



CLINICAL REPORT

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

Yukitomo Ishi¹, Takumi Era², Sayaka Yuzawa³, Michinari Okamoto¹, Ryosuke Sawaya¹, Hiroaki Motegi¹, Shigeru Yamaguchi¹, Shunsuke Terasaka^{1,4}, Kiyohiro Houkin¹, Miki Fujimura¹

¹Department of Neurosurgery, Faculty of Medicine and Graduate School of Medicine, Hokkaido University, North 15 West 7, Kita-ku, Sapporo 060-8638, Japan

²Department of Cell Modulation, Institute of Molecular Embryology and Genetics, Kumamoto University, 2-2-1 Honjo, Chuo-ku, Kumamoto 860-0811, Japan

³Department of Diagnostic Pathology, Asahikawa Medical University Hospital, Asahikawa, Japan

⁴Department of Neurosurgery, Kashiwaba Neurosurgical Hospital, Sapporo, Japan

Grant number: JSPS KAKENHI grant number, JP 19K09519

Corresponding Author: Shigeru Yamaguchi, MD

Department of Neurosurgery, Hokkaido University Graduate School of Medicine

North 15 West 7, Kita-ku, Sapporo 060-8638, Japan

Phone: (+81)11-706-5987, Fax: (+81)11-708-7737

E-mail: <u>yama-shu@med.hokudai.ac.jp</u>

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

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.

Clinical Report

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.

Acknowledgments

This research was supported by JSPS KAKENHI (grant number JP 19K09519). We would like to thank Yumiko Soejima (Department of Cell Modulation, Institute of Molecular Embryology and Genetics, Kumamoto University) for providing technical assistance for iPSC generation. The authors would like to thank Enago (<u>www.enago.jp</u>) for the English language review.

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.

References

- Asthagiri, A. R., Parry, D. M., Butman, J. A., Kim, H. J., Tsilou, E. T., Zhuang, Z., & Lonser,
 R. R. (2009). Neurofibromatosis type 2. *Lancet*, 373(9679), 1974-1986.
 doi:10.1016/S0140-6736(09)60259-2
- Evans, D. G., Hartley, C. L., Smith, P. T., King, A. T., Bowers, N. L., Tobi, S., Wallace, A. J., Perry, M., Anup, R., Lloyd, S. K. W., Rutherford, S. A., Hammerbeck-Ward, C., Pathmanaban, O. N., Stapleton, E., Freeman, S. R., Kellett, M., Halliday, D., Parry, A., Gair, J. J., Axon, P., Laitt, R., Thomas, O., Afridi, S. K., Obholzer, R., Duff, C., Stivaros, S. M., Vassallo, G., Harkness, E. F., & Smith, M. J. (2020). Incidence of mosaicism in 1055 de novo NF2 cases: much higher than previous estimates with high utility of next-generation sequencing. *Genet Med*, 22(1), 53-59. doi:10.1038/s41436-019-0598-7
- Evans, D. G., Huson, S. M., Donnai, D., Neary, W., Blair, V., Newton, V., & Harris, R. (1992). A clinical study of type 2 neurofibromatosis. *Q J Med*, *84*(304), 603-618.
- Evans, D. G., Ramsden, R. T., Shenton, A., Gokhale, C., Bowers, N. L., Huson, S. M., Pichert, G., & Wallace, A. (2007). Mosaicism in neurofibromatosis type 2: an update of risk based on uni/bilaterality of vestibular schwannoma at presentation and sensitive mutation analysis including multiple ligation-dependent probe amplification. *J Med Genet*, 44(7), 424-428. doi:10.1136/jmg.2006.047753
- Fiacco, E., Alowaysi, M., Astro, V., & Adamo, A. (2020). Derivation of two naturally isogenic iPSC lines (KAUSTi006-A and KAUSTi006-B) from a mosaic Klinefelter

Syndrome patient (47-XXY/46-XY). *Stem Cell Res, 49*, 102049. doi:10.1016/j.scr.2020.102049

- Halliday, D., Emmanouil, B., Pretorius, P., MacKeith, S., Painter, S., Tomkins, H., Evans, D.
 G., & Parry, A. (2017). Genetic Severity Score predicts clinical phenotype in NF2. J Med Genet, 54(10), 657-664. doi:10.1136/jmedgenet-2017-104519
- Hanatani, T., & Takasu, N. (2020). CiRA iPSC seed stocks (CiRA's iPSC Stock Project). Stem Cell Res, 50, 102033. doi:10.1016/j.scr.2020.102033
- Hanemann, C. O., Diebold, R., & Kaufmann, D. (2007). Role of NF2 haploinsufficiency in NF2-associated polyneuropathy. *Brain Pathol*, 17(4), 371-376. doi:10.1111/j.1750-3639.2007.00086.x
- Kawasaki, Y., Oda, H., Ito, J., Niwa, A., Tanaka, T., Hijikata, A., Seki, R., Nagahashi, A., Osawa, M., Asaka, I., Watanabe, A., Nishimata, S., Shirai, T., Kawashima, H., Ohara, O., Nakahata, T., Nishikomori, R., Heike, T., & Saito, M. K. (2017). Identification of a high-frequency somatic nlrc4 mutation as a cause of autoinflammation by pluripotent cell-based phenotype dissection. *Arthritis Rheumatol, 69*(2), 447-459. doi:10.1002/art.39960
- Maeda, H., Chiyonobu, T., Yoshida, M., Yamashita, S., Zuiki, M., Kidowaki, S., Isoda, K.,
 Yamakawa, K., Morimoto, M., Nakahata, T., Saito, M. K., & Hosoi, H. (2016).
 Establishment of isogenic iPSCs from an individual with SCN1A mutation mosaicism as a model for investigating neurocognitive impairment in Dravet syndrome. *J Hum Genet*, *61*(6), 565-569. doi:10.1038/jhg.2016.5
- Moyhuddin, A., Baser, M. E., Watson, C., Purcell, S., Ramsden, R. T., Heiberg, A., Wallace,A. J., & Evans, D. G. (2003). Somatic mosaicism in neurofibromatosis 2: prevalenceand risk of disease transmission to offspring. *J Med Genet*, 40(6), 459-463.

doi:10.1136/jmg.40.6.459

- Paganini, I., Mancini, I., Baroncelli, M., Arena, G., Gensini, F., Papi, L., & Sestini, R. (2014). Application of COLD-PCR for improved detection of NF2 mosaic mutations. *J Mol Diagn*, 16(4), 393-399. doi:10.1016/j.jmoldx.2014.02.007
- Petrilli, A. M., & Fernández-Valle, C. (2016). Role of Merlin/NF2 inactivation in tumor biology. Oncogene, 35(5), 537-548. doi:10.1038/onc.2015.125
- Smith, M. J., Bowers, N. L., Bulman, M., Gokhale, C., Wallace, A. J., King, A. T., Lloyd, S. K., Rutherford, S. A., Hammerbeck-Ward, C. L., Freeman, S. R., & Evans, D. G. (2017). Revisiting neurofibromatosis type 2 diagnostic criteria to exclude LZTR1-related schwannomatosis. *Neurology*, 88(1), 87-92. doi:10.1212/wnl.00000000003418
- Soga, M., Ishitsuka, Y., Hamasaki, M., Yoneda, K., Furuya, H., Matsuo, M., Ihn, H., Fusaki, N., Nakamura, K., Nakagata, N., Endo, F., Irie, T., & Era, T. (2015). HPGCD outperforms HPBCD as a potential treatment for Niemann-Pick disease type C during disease modeling with iPS cells. *Stem Cells, 33*(4), 1075-1088. doi:10.1002/stem.1917
- Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., & Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*, 131(5), 861-872. doi:10.1016/j.cell.2007.11.019
- Teranishi, Y., Miyawaki, S., Hongo, H., Dofuku, S., Okano, A., Takayanagi, S., Ota, T., Yoshimura, J., Qu, W., Mitsui, J., Nakatomi, H., Morishita, S., Tsuji, S., & Saito, N. (2021). Targeted deep sequencing of DNA from multiple tissue types improves the diagnostic rate and reveals a highly diverse phenotype of mosaic neurofibromatosis type 2. *J Med Genet*, 58(10), 701-711. doi:10.1136/jmedgenet-2020-106973

- van der Wal, E., den Hamer, B., van der Vliet, P. J., Tok, M., Brands, T., Eussen, B., Lemmers, R., Freund, C., de Klein, A., Buijsen, R. A. M., van Roon-Mom, W. M. C., Tawil, R., van der Maarel, S. M., & de Greef, J. C. (2019). Generation of genetically matched hiPSC lines from two mosaic facioscapulohumeral dystrophy type 1 patients. *Stem Cell Res, 40*, 101560. doi:10.1016/j.scr.2019.101560
- Yamanaka, S. (2020). Pluripotent stem cell-based cell therapy-promise and challenges. *Cell Stem Cell*, 27(4), 523-531. doi:10.1016/j.stem.2020.09.014
- Yuzawa, S., Nishihara, H., Yamaguchi, S., Mohri, H., Wang, L., Kimura, T., Tsuda, M., Tanino, M., Kobayashi, H., Terasaka, S., Houkin, K., Sato, N., & Tanaka, S. (2016).
 Clinical impact of targeted amplicon sequencing for meningioma as a practical clinical-sequencing system. *Mod Pathol, 29*(7), 708-716. doi:10.1038/modpathol.2016.81

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)	#1	#2	#3	#4	#5	PC1	PC2	NC
SeV_1st	6						-	
SeV_nested							-	
β-actin		-	-					
NANOG	-		-	-	-			
c-MYC			-	-			-	
GDF3	-	-	-	I	1	-	-	
KLF4	-	-	-		-			8-34
SALL4	-							, 2000 - A
OCT3/4	-							
REX1	-	-	-	-	-	-	-	
SOX2	-							
DNMT3b	-	-	-	-	-	-	-	



