Preparation of the standard cell lines for reference mutations in cancer gene-panels by genome editing in HEK 293 T/17 cells

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Abstract

Background: Next Generation Sequencer (NGS) is a powerful tool for a high-throughput sequencing of human genome. It is important to ensure reliability and sensitivity of the sequence data for a clinical use of the NGS. Various cancer-related gene panels such as Oncomine™ or NCC OncoPanel have been developed and used for clinical studies. Because these panels contain multiple genes, it is difficult to ensure the performance of mutation detection for every gene. In addition, various platforms of NGS are developed and their cross-platform validation has become necessity. In order to create mutant standards in a defined background, we have used CRISPR/Cas9 genome-editing system in HEK 293 T/17 cells.

Results: Cancer-related genes that are frequently used in NGS-based cancer panels were selected as the target genes. Target mutations were selected based on their frequency reported in database, and clinical significance and on the applicability of CRISPR/Cas9 by considering distance from PAM site, and off-targets. We have successfully generated 88 hetero- and homozygous mutant cell lines at the targeted sites of 36 genes representing a total of 125 mutations.

Conclusions: These knock-in HEK293T/17 cells can be used as the reference mutant standards with a steady and continuous supply for NGS-based cancer panel tests from the JCRB cell bank. In addition, these cell lines can provide a tool for the functional analysis of targeted mutations in cancer-related genes in the isogenic background.

Keywords: Next generation sequencer, Genome editing, CRISPR/Cas9, Mutant standard, Cancer gene panel

Introduction

Innovative advances in DNA sequencing technology have deepened the understanding of cancer genetic abnormalities and accumulated huge volumes of data on genetic abnormalities in various human cancers [1–4]. On the other hand, the so-called molecular targeted drugs, targeting specific cancer-related genes, have been developed based on the genetic alterations observed in human cancers [5]. Diagnosis of a genetic abnormality is becoming indispensable for deciding whether to administer a drug, which is known as the companion diagnosis [6–8] for the molecular targeted drugs in which genetic abnormalities and therapeutic action of the drugs are clearly linked. Last few years have witnessed increasing the clinical application of the so-called “cancer gene panels” [9, 10] for a comprehensive analysis of multiple genetic abnormalities. At the same time application of the next-generation sequencers (NGS), which is expected as a useful tool for realizing genomic medicine on human cancer, is gaining popularity. For its clinical application, however, it is necessary to ensure the reliability and sensitivity of the sequence data for multiple genes [11, 12]. But it is difficult to guarantee the performance of mutation detection for all installed genes in the comprehensive cancer gene panel. Such cancer panel tests consist of multiple steps, including sample preparation, nucleic acid extraction, library preparation for sequence analysis, and hardware (NGS) and software for sequence determination, and making it difficult to ensure validity for all steps. Therefore a standard method to validate the process of the whole diagnosis system by using the standard material is advocated [13].

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Synthetic DNAs of known sequence can be used as a standard for validation of a DNA sequencer itself [14], but it is desirable to use a pathological specimen closer to real clinical samples in order to validate the whole processes of diagnosis including sample preparation [11]. When the number of gene to be sequenced is limited, a portion of the clinical specimen can be preserved and used as the reference material for the specific gene, but it is difficult to prepare such standards for all the genes in the panel. Further, for several genetic diseases, authentic clinical samples are not available due to rarity of the mutation. Therefore, development of similar standard products is desired to serve as useful tools in the development and validation of test systems. In addition, from the viewpoint of steady supply, purity (heterogeneity), and coverage of mutations, it is difficult to utilize FFPE specimen in a long run. Therefore, the established cell line, which is considered to be closer to clinical samples than synthetic DNA has been proposed to maintain a steady supply of the homogeneous and more reliable reference material covering whole range of mutations in the cancer gene panels. If the cultured cells can be used, it is also possible to combine variety of gene mutations, which is particularly desirable as a standard for the cancer gene panels.

In order to create such mutant standards for versatile cancer-related genes, we have used a genome-editing technology with CRISPR/Cas9 [15, 16], which is recently getting popular, and tried to integrate known mutations of interest into a defined cell line. The human embryonic kidney derived cell line, HEK 293 T/17 cell, which is frequently used for genome-editing because of a high efficiency, was used. Details of pathogenic and high frequency mutations reported in the COSMIC database [17] was retrieved, guide RNAs were designed for those mutations and appropriate knock-in strains were created by genome editing. In this process, since the clinical application of NCC Oncopanel [18], developed by the National Cancer Research Center, was progressed, we decided to select those genes that are included in the COSMIC database (REF [19]) were selected as the candidate genes and details on mutations reported for those genes were searched in the COSMIC database [1]. In the candidate genes, those mutations which are reported to be pathogenic and found with higher frequency were selected and also considering adjacent Cas9-target sequence of PAM (protospacer adjacent motif) site (3’ NGG) for Cas9 cleavage. Then the guide RNA (gRNA), which contains a complementary sequence of the targeting site, was designed for the mutation. The possibility of off-target effect was checked by the GGGnome software and those with higher off-targets were avoided.

### Genome-editing by CRISPR/Cas9

For the genome-editing, the DNA-directed RNA-guided endonuclease (RGEN) system (TakaraBio) was used [20]. Designed gRNA sequence was integrated into the expression vector under the U6 promoter (pRGEN_U6_SG). Cas9 endonuclease was integrated into the expression vector under the CMV promoter (pRGEN-Cas9-CMV). These plasmids were transfected into E.coli and purified by the NucleoBond Xtra Midi EF (Macherey-Nagel). The 71–78 bp long single-stranded oligonucleotide (ssODN) (100 pmol) was transfected along pRGEN_U6_SG (0.17 μg) and pRGEN-Cas9-CMV (0.25 μg) vectors into 1.75 × 10^5 HEK 293 T/17 cells by using TransIT-X2 reagent (Mirus Bio). Then the cells were cultured in 24 well tissue culture plates for 3 days. As an alternative method for 4 genes (AKT3, BIM, IGF2 and MYCN), gRNA was prepared by in vitro transcription (IVT) using Guide-it for Cas9 protein by the Neon Transfection System (Thermo Fisher Scientific) with two pulses of 1100 V and 20 ms.

Regarding the method of introducing the Cas9, a transfection of the Cas9 proteins as a complex with gRNA (RNP) was used in the later experiment [20], instead of the standard expression vector method.

### Performance of gRNA assessed by T7E1 assay

After 3 days, a portion of the culture cells was subjected to the T7E1 assay [21]. Genomic DNA was prepared from the cells by the Nucleospin Tissue Kit (Macherey-Nagel) and the targeted region was PCR amplified by Tks Gflex DNA Polymerase (TakaraBio) with the appropriate primers (94°C for 1 min; 98°C for 10s, 60°C for 20 ms.

In this article, we describe introduce the creation of the genome edited strains and their properties, and discuss about their usage including a use for the comprehensive standard for the NCC OncoPanel.

### Materials and methods

#### Cells

The human embryonic kidney HEK 293 T/17 cells were obtained from ATCC Manassas, VA, USA. The cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Thermo Fisher Scientific) and 1% penicillin-streptomycin (Thermo Fisher Scientific). Absence of mycoplasma was checked by the MycoAlert Mycoplasma Detection Kit (Lonza) occasionally.

#### Selection of the target mutations in cancer-related genes

Cancer-related genes that are frequently used in the cancer gene panels, or those found in the Japanese mutation database (REF [19]) were selected as the candidate genes and details on mutations reported for those genes were searched in the COSMIC database [1]. In the candidate genes, those mutations which are reported to be pathogenic and found with higher frequency were selected and also considering adjacent Cas9-target sequence of PAM (protospacer adjacent motif) site (3’ NGG) for Cas9 cleavage. Then the guide RNA (gRNA), which contains a complementary sequence of the targeting site, was designed for the mutation. The possibility of off-target effect was checked by the GGGnome software and those with higher off-targets were avoided.
15 s, 68 °C for 30 s) for 30 cycles; kept at 4°C in the TP600 thermal cycler (TakaraBio). The PCR products (20 μl) were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel), heat denatured and re-annealed by the TP600 thermal cycler (94°C for 2 min; 85°C for 1 s; 30°C for 10 min; kept at 4°C). Then 2 μl of T7 Endonuclease 1 (10 U/μl; New England Biolabs) was added and incubated at 37°C for 30 min. The reaction was stopped by adding 1 μl of 0.5 M EDTA. The digested DNA fragments were analyzed by the Agilent 1000 kit in 2100 Bioanalyzer (Agilent Technologies). When the digested fragment was detected, the cells were subjected further to the single cell cloning.

Cloning and screening of the targeted mutations
The cells were harvested by 0.25% Trypsin-EDTA (Thermo Fisher Scientific) to make a single cell suspension and plated into 96 Half Area Well Clear Flat Bottom TC-Treated Microplate (Sigma-Aldrich) at a density of 0.4 cells/well. Then cells were expanded by transferring into 24 well tissue culture plates and each clone was stored in CELLBANKER1 at ~80°C until DNA sequence analysis and subsequent stoke.

For the sequence analysis, the cell pellet was subjected to the Single Prep reagent for DNA (TakaraBio) and the prepared genomic DNA was used for the amplification of targeted regions by the corresponding primers. After purification of the PCR products by Sephadex G-50 Fine column (GE Healthcare), DNA sequence was analyzed by the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) with the forward primer for amplification.

Results
The selected target genes and mutation sites are summarized in Table 1. Although the panel of 39 target genes was selected mainly from NCC OncoPanel [22], those with more overlap with other similar cancer gene panels (Oncomine [23], Illumina Trusight tumor 26 [24], Personalis [25], and Ion Ampliseq Cancer Hotspot panel [26]) were also included. Target site for mutation was basically selected from the COSMIC mutations registered with higher frequency and pathogenicity and with a consideration of the Japanese database (NBDC) [19]. Then the gRNA was designed for the target site closest to a PAM site and with less chance of off-target. Number of 1–3 mismatch sites in genome was screened by the GG Gn software. Single strand oligo DNA (ssODN) adjacent to the target site was prepared with a length of 71–78 bp. Based on the gRNA sequence, expression vector pRGEN_U6_SG was constructed for each target gene which was co-transfected with the Cas9 expression vector pRGEN-Cas9-CMV and ssODN.

Effectiveness of gRNA to cut the targeted DNA sequence was monitored by the T7E1 assay using the genomic DNA isolated from the transfected cells. Figure 1 shows results of T7E1 assay for KRAS, NRAS, PIK3CA, PTEN, BRAF and TP53 genes. When the predicted DNA fragments were obtained in the T7E1 assay, the cells were taken for further cloning. In the case of negative results, the gRNA was re-designed or the target gene was changed.

Finally 36 out of 39 target genes were preceded for colony isolation and sequence determination. Due to the inability of using a marker for knock-in mutant selection, more than 150 clones were screened for each gene by the direct Sanger sequencing of PCR products (Fig. 2). Because HEK 293 T/17 cell is having a near triploid karyotype with various copy number among the whole chromosome region, it was difficult to fully diagnose the sequence spectrum. The targeted event was judged as homozygote or heterozygote as shown in Table 2. The latter includes other than 1:1 ratio depending on the chromosomal location but the exact number of the mutant allele was not determined. Because of the unstable karyotype, the exact copy number should be validated before being used as a reference cell line. It can be thought that the copy number of whole population is stable at least within a few passages during a reference preparation.

Together with the targeted knock-in event, many dele-tion mutations were observed around the targeted site; it happened simultaneously in different allele in some cases. Off-target mutations near the targeted site were also observed in few cases. An improvement in editing efficiency was seen in RNP experiments (average success rate increased to 10.2 from 3.5, p = 0.0055 by T-test) (Table 2).

Initially five clones each for 6 genes (NRAS, KRAS, PIK3CA, PTEN, TP53 and BRAF) and then two clones each for the rest were selected and stored after expansion of the culture. Out of 36 genes taken for trial, the targeted mutation (either homo or hetero) could be obtained for 33 genes with more than 90% success rate. The mutant clones having more variations in each gene were selected. Finally, a panel of 88 isolated clones of HEK 293 T/17 cells representing 125 mutations in total was produced including some non-targeted mutants (Table 3).

Discussion
With the progress made with the genome editing technology using CRISPR/Cas9, it has become possible to modify the genes of interest at relatively easy manner. We applied this technique to prepare a panel of cell lines in which a known gene mutation has been introduced into a target site to use as a standard reference material.
| Gene     | NCC Oncopanel (ver 2) | Thermo Fisher Oncorome Dx Test | Personalis ACE CancerPlus™ Test | Illumina TruSight Tumor 26 | Ion AmpliSeq Cancer Hotspot Panel v2 | No of mutations in COSMIC | COSMIC_ID     | CDS Mutation | FATHMM Pathogenic (score) | gRNA sequence from PAM | (No of mismatch) |
|----------|-----------------------|-------------------------------|-------------------------------|-----------------|--------------------------------|---------------------------|----------------|--------------|--------------------------|----------------------|------------------|
| AKT1     | *                     | *                             | *                             | *               | 975                             | COSM33765                 | 49G > A       | Pathogenic   | (score 1.00)              | CACCACTCCGCACG       | 1                |
| AKT3     | *                     |                               |                               | *               | 269                             | COSM242892                 | 232C > A      | Pathogenic   | (score 0.99)              | TCACTATACGACTG       | 1                |
| ALK      | *                     |                               |                               | *               | 1522                            | COSM28056                 | 3824G > A     | Pathogenic   | (score 0.98)              | TACTCACCCTAGTGATGCTGG | 4                |
| BAP1     | *                     |                               |                               | *               | 1221                            | COSM110721                 | 178C > T      | Pathogenic   | (score 0.98)              | CCAAGTGATAGACCTGGTCG | 5                |
| BCL2L11 (BIM) | *                     |                               |                               | *               | 116                             | COSM389356                 | 585G > C      | Pathogenic   | (score 0.98)              | GTCTACAGTGAAATCTGG   | 1                |
| BRAF     | *                     |                               |                               | *>              | 27630                           | COSM476                   | 1799T > A     | Pathogenic   | (score 0.99)              | TAGCTACAGTGAGGGTCTGG | 12               |
| CDK4     | *                     |                               |                               | *               | 101                             | COSM1677139               | 70C > T       | Pathogenic   | (score 0.99)              | AGTGGGCCACGTGGAGGGT | 2                |
| CDKN2A (p16) | *                     |                               |                               | *               | 5911                            | COSM12475                 | 238C > T      | Pathogenic   | (score 0.88)              | GGCGACGCTGGAGGGTGGAGGCCTGG | 2                |
| CTNNB1 (β-catenin) | *                     |                               |                               | *               | 7307                            | COSM5664                 | 121A > G      | Pathogenic   | (score 0.98)              | CAGAGAAGGAGACGAGTGG | 2                |
| DNMT3A   | *                     |                               |                               | *               | 3679                            | COSM52944                 | 2645G > A     | Pathogenic   | (score 0.98)              | CGTCTCGACGTGGAGGGTGG | 6                |
| ERBB2 (HER2) | *                     |                               |                               | *               | 1596                            | COSM48358                 | 929C > T      | Pathogenic   | (score 0.97)              | CAGGGGCCGAGACGAGGTCG | 6                |
| ERBB3    | *                     |                               |                               | *               | 900                             | COSM20710                 | 310G > A      | Pathogenic   | (score 0.88)              | ACCATTGCCCACCTCGCCG | 4                |
| EZH2     | *                     |                               |                               | *               | 1273                            | COSM37028                 | 1937A > T     | Pathogenic   | (score 0.99)              | GAATTCTCATCTGAAATACTG | 7                |
| FBXW7    | *                     |                               |                               | *               | 1972                            | COSM22975                 | 1513C > T     | Pathogenic   | (score 0.94)              | TGCCATCATATTGAAACACAG | 2                |
| FGFR3    | *                     |                               |                               | *               | 4354                            | COSM715                   | 746C > G      | Pathogenic   | (score 0.96)              | CTGCAGGATGCAGCGGCGGTGG | 1                |
| FOXL2    | *                     |                               |                               | *               | 933                             | COSM33661                 | 402C > G      | Pathogenic   | (score 0.95)              | CTTCCTGAAACGTGCTGG | 5                |
| HRAS     | *                     |                               |                               | *               | 2023                            | COSM3502                 | 183G > T      | none         | (score 0.53)              | CATCCTGATACCCTGCCG | 2                |
| Gene | NCC Oncopanel (ver 2) | Thermo Fisher Oncomine Dx Test | Personalis ACE CancerPlus™ Test | Illumina TruSight Tumor 26 | Ion AmpliSeq Cancer Hotspot Panel v2 | No of mutations in COSMIC | COSMIC_ID | CDS Mutation | FATHMM (score) | gRNA sequence | Distance from PAM | (No of mismatch) |
|------|----------------------|-------------------------------|--------------------------------|-----------------------------|---------------------------------|--------------------------|-----------|--------------|----------------|---------------|----------------|----------------|
| IDH2 | *                    | *                             | *                              | *                           | *                               | 2430                     | COSM33733 | 515G > A   | Pathogenic (score 0.99) | CCAAGCCCCATCA CCATTGCGCAGG | 2               | 1 1 4 86 |
| IGF2 | *                    |                               |                                |                             |                                 | 132                      | COSM1561457 | 293C > T   | Pathogenic (score 0.97) | ACCCTCAACCGGA AGCACGGTCGGG | 2               | 1 0 0 1 |
| JAK2 | *                    | *                             | *                              |                             |                                 | 50,556                   | COSM12600  | 1849G > T  | Pathogenic (score 0.94) | AATTAGGGAGATAT GTGTCGTGGG | 8               | 1 1 1 75 |
| KIT  | *                    | *                             | *                              | *                           | *                               | 8856                     | COSM1314  | 2447A > T  | Pathogenic (score 0.99) | AGAAATCATCCTTG ATGTCTCTGGG | 7               | 1 1 1 133 |
| KNSRN | *                   |                               |                                |                             |                                 | 118                      | COSM140056 | 71C > T   | Neutral (score 0.00)    | GTAAGCTAGGGGG AAGTGGGTGGG | 1               | 1 1 1 28 |
| KRAS | *                    | *                             | *                              | *                           | *                               | 43,548                   | No ID     | 124G > A   | –               | CTTGGTAGTTG GAGCTGGTGG | 4               | 1 1 1 46 |
| MAGOH | *                   |                               |                                |                             |                                 | 43                       | COSM535605 | 410T > C   | Pathogenic (score 0.99) | TGTGGTGTCTCTCA GTCTTTATGG | 4               | 1 2 3 76 |
| MAP 2 K1 | *              | *                             | *                              | *                           | *                               | 496                      | COSM235614 | 370C > T   | Pathogenic (score 0.99) | CCATAAGAAAGCC AGCATGACGGG | 1               | 1 4 4 10 |
| MAPK1 | *                    |                               |                                |                             |                                 | 173                      | COSM461148 | 964G > A   | Pathogenic (score 0.98) | GTAACGACCCGG AGTGAGCGGG | 4               | 1 1 1 2 |
| MDM2 | *                    |                               |                                |                             |                                 | 110                      | COSM431747 | 994C > T   | Pathogenic (score 0.91) | AGGAAGCCAAAT CTCAAGACGGG | 6               | 1 1 2 25 |
| MET  | *                    | *                             | *                              | *                           | *                               | 1142                     | COSM707   | 3029C > T  | Pathogenic (score 0.98) | TTGAAACATTTT CTGATGTGGG | 5               | 1 1 2 78 |
| MTOR | *                    | *                             | *                              |                             |                                 | 1195                     | COSM20417 | 6644C > A  | Pathogenic (score 1.00) | CCAATGGCCCCA CATCTCTTCGG | 8               | 1 1 1 12 |
| MYCN | *                    |                               |                                |                             |                                 | 305                      | COSM35624  | 131C > T   | Pathogenic (score 0.96) | CTCCTAGAGTCC TCCCCGGG | 1               | 1 0 0 24 |
| NOTCH1 | *               | *                             | *                              | *                           | *                               | 3921                     | COSM12771 | 4799T > C  | Pathogenic (score 0.99) | CACGCTTGTGCTGG CAGCACGGGG | 9               | 1 1 1 53 |
| NRAS | *                    | *                             | *                              | *                           | *                               | 6676                      | COSM5930625 | 112G > A  | Pathogenic (score 0.99) | AAACCTGGTGATG GTTGGAGCGGG | 1               | 1 1 1 67 |
| PDGFRα | *              | *                             | *                              | *                           | *                               | 2354                      | COSM736   | 2525A > T  | Pathogenic (score 0.99) | CGAATCATGCTG CATGTCTCTGGG | 7               | 1 1 1 24 |
| PIK3CA | *              | *                             | *                              | *                           | *                               | 13613                     | COSM775   | 3140A > G  | Pathogenic (score 0.98) | ATGAATGATGCA CATCATGGTGGG | 1               | 1 0 1 56 |
| PTCH1 | *                    | *                             | *                              |                             |                                 | 1331                     | COSM1638394 | 3944C > T  | Pathogenic (score 1.00) | AGGCCTCTCTGGG GCCTGCTTAGG | 1               | 1 1 1 7 |
Table 1 List of targeted genes, site of mutations, and design of gRNA (Continued)

| Gene       | NCC | Oncopanel (ver 2) | Thermo Fisher Oncornone Dx Test | Personalis ACE CancerPlus™ Test | Illumina TruSight Tumor 26 | Ion AmpliSeq Cancer Hotspot Panel v2 | No of mutations in COSMIC | COSMIC_ID | Mutation | FATHMM | gRNA sequence (No of mismatch) | Distance from PAM | (No of mismatch) |
|------------|-----|-------------------|--------------------------------|--------------------------------|----------------------------|------------------------------------|--------------------------|------------|----------|--------|--------------------------------|-----------------|-----------------|
| PTEN       | *   | *                 | *                               | *                               | *                          | 4778                               | No ID                    | 697C > T   | –        | AACTGTCCTTCCC GTCGTGTGGG       | 7               | 1 0 1 7         |
| SMO        | *   | *                 | *                               | *                               |   | 593                               | COSM216037                | 1234C > T  | Pathogenic (score 0.96) | AGCCCTCCACGA TGAGCACCAGG | 8               | 1 1 1 102        |
| STAT3      | *   | *                 |                                  |                                  |   | 805                               | COSM1555748               | 1919A > T  | Pathogenic (score 0.97) | TTCAGCTGCTGCT TGTGATAGG | 5               | 1 1 6 92         |
| TP53(p53)  | *   | *                 | *                               | *                               |   | 39,196                             | COSM10662                 | 743G > A   | Pathogenic (score 0.98) | GCATGGCCGGCA TGAACCGGAGG | 5               | 1 0 0 3          |
Fig. 1 Results of T7E1 assay. After transfection of expression vectors pRGEN_U6_SG, pRGEN-Cas9-CMV and ssODN corresponding to the target genes (KRAS, NRAS, PIK3CA, PTEN, BRAF and TP53), genomic DNA was isolated from the transfected cells. The target region was amplified by PCA with corresponding primers. After denature and re-annealing of the PCR product, it was digested by T7 endonuclease I (T7E1) which cut mismatched DNA fragments. Cleaved band suggests an introduction of mutations by ssODN.

Fig. 2 Confirmation of the TP53 743 G > A mutation by Sanger sequencing. Existence of the targeted knock-in mutation was checked for each isolated clones by the Sanger sequencing after PCR amplification of the target region. Successful examples for TP53 743 G > A mutation are shown. Both homo and hetero knock-in mutants were obtained.
for genetic diagnosis. Since genome editing was carried out for 39 genes, the basic data obtained in this study could be analyzed for improving genome editing efficiency. The designing of gRNA can be discussed as an important factor in genome editing.

The PAM site, which is a cleavage site by Cas9 protein, is known to be important to initiate genome editing, and it is desirable to select the mutation of interest close to the PAM site [27]. In the standard method of using a Cas9 expression vector, it was predicted that it is desirable to select the site of the target mutation closer to the PAM site. But our data demonstrated that even when it is designed in the vicinity of a PAM site, the genome editing efficiency is not necessarily high, and we could get the mutants even when it is far from the PAM site (up to 9 bp). Therefore, genome editing efficiency was not affected so much by the distance from the PAM site (Correlation coefficient between bp from PAM site and genome editing efficiency: r = 0.7).

### Table 2 Targetted mutations and recoveries of the knock-in mutants

| Method | Gene | Mutation | bp from PAM | Total Clones | Homo Knock-in | Hetero Knock-in | Targeted Rate(%) |
|--------|------|----------|-------------|--------------|---------------|----------------|------------------|
| Vector | AKT1 | 49 G > A | 1 | 177 | 0 | 0 | 0.0 |
| Vector | ALK | 3824 G > A | 4 | 190 | 4 | 5 | 6.8 |
| Vector | BAP1 | 178 C > T | 5 | 183 | 0 | 12 | 6.6 |
| Vector | CDKN2A | 238 C > T | 2 | 156 | 5 | 2 | 7.7 |
| Vector | CTNNB1 | 121 A > G | 6 | 164 | 1 | 0 | 1.2 |
| Vector | DNMT3A | 264 G > A | 6 | 149 | 0 | 1 | 0.7 |
| Vector | ERBB3 | 310 G > A | 4 | 171 | 0 | 1 | 0.6 |
| Vector | EZH2 | 1937 A > T | 7 | 153 | 4 | 0 | 5.2 |
| Vector | FBXW7 | 1513 C > T | 2 | 167 | 0 | 0 | 0.0 |
| Vector | FGFR3 | 746 C > G | 1 | 167 | 0 | 1 | 0.6 |
| Vector | HRAS | 183 G > T | 2 | 172 | 2 | 5 | 5.2 |
| Vector | IDH2 | 515 G > A | 2 | 155 | 0 | 7 | 4.5 |
| Vector | JAK2 | 1849 G > T | 8 | 164 | 3 | 1 | 4.3 |
| Vector | KIT | 2447 A > T | 7 | 172 | 3 | 12 | 10.5 |
| Vector | KNSTRN | 71 C > T | 1 | 161 | 1 | 0 | 1.2 |
| Vector | KRAS | 35 G > A | 4 | 160 | 0 | 2 | 1.3 |
| Vector | MAGOH | 410 T > C | 4 | 160 | 0 | 5 | 3.1 |
| Vector | MAP_2 K1 | 370 C > T | 1 | 177 | 7 | 0 | 1.1 |
| Vector | MAPK1 | 964 G > A | 4 | 169 | 0 | 0 | 0.0 |
| Vector | MDM2 | 994 C > T | 6 | 171 | 0 | 2 | 1.2 |
| Vector | MET | 3029 C > T | 5 | 154 | 1 | 1 | 1.9 |
| Vector | MTOR | 6644 C > A | 8 | 182 | 2 | 9 | 7.1 |
| Vector | NOTCH1 | 4799 T > C | 9 | 167 | 0 | 2 | 1.2 |
| Vector | NRAS | 35 G > A | 1 | 157 | 2 | 0 | 2.5 |
| Vector | PDGFRα | 2525 A > T | 7 | 176 | 7 | 6 | 11.4 |
| Vector | PIK3CA | 3140 A > G | 1 | 161 | 0 | 1 | 0.6 |
| Vector | PTEN | 697 C > T | 7 | 156 | 0 | 1 | 0.6 |
| Vector | SMO | 1234 C > T | 8 | 157 | 8 | 7 | 14.6 |
| Vector | STAT3 | 1919 A > T | 5 | 161 | 0 | 1 | 0.6 |
| Vector | TPS3 | 743 G > A | 5 | 159 | 2 | 2 | 3.8 |
| Protein | BRAF | 1817 G > A | 1 | 172 | 1 | 16 | 10.5 |
| Protein | AKT3 | 232 C > A | 2 | 192 | 0 | 5 | 2.6 |
| Protein | BIM | 585 G > C | 1 | 192 | 6 | 9 | 10.9 |
| Protein | IGF2 | 293 C > T | 2 | 192 | 0 | 6 | 3.1 |
| Protein | MYCN | 1132 G > A | 1 | 192 | 10 | 26 | 24.0 |
| Target Gene | JCRB Cell Name (ID) | JCRB Cell ID | Mutation detail a |
|-------------|---------------------|----------------|-------------------|
| AKT1        | 49 G>A              | 293T-AKT1-1   | delTG(43-44) Homo |
| AKT3        | 232C>A              | 293T-AKT3-1   | 232C>A(COSM242892) Hetero+del231-238(8bp) Hetero+236delG&235T>A Homo |
| ALK         | 3824 G>A            | 293T-ALK-1    | 3824G>A(COSM28056) Homo |
| BAP1        | 178 C>T             | 293T-BAP1-1   | 178C>T(COSM110721) Hetero |
| BIM         | 585G>C              | 293T-BIM-1    | 585G>C(COSM389356) Homo+590 insT Hetero |
| BRAF        | 1817G>A             | 293T-BRAF-1   | 1817G>A(COSM1137) Homo |
| CDKN2A      | 238 C>T             | 293T-CDKN2A-1 | 238C>T(COSM12475) Homo |
| CTNNB1      | 121 A>G             | 293T-CTNNB1-1 | 121A>G(COSM5664) Homo+del116-9&112G>A Hetero+del114-9 Hetero |
| DNMT3A      | 2645 G>A            | 293T-DNMT3A-1 | 2645G>A(COSM52944) Homo |
| ERBB3       | 310 G>A             | 293T-ERBB3-1  | 306insC Homo |
| EZH2        | 1937 A>T            | 293T-EZH2-1   | 1937A>T(COSM37028) Homo |
| FBXW7       | 1513 C>T            | 293T-FBXW7-1  | 1518insT Homo |
| FGFR3       | 746 C>G             | 293T-FGFR3-1  | 752insA Homo |
| HRAS        | 183 G>T             | 293T-HRAS-1   | 183G>T(COSM5022) Homo |
| IDH2        | 515 G>A             | 293T-IDH2-1   | 515G>A(COSM33733) Homo+512G>A(COSM86960) Homo |
| IGF2        | 293C>T              | 293T-IGF2-1   | 293C>T(COSM1561457) Homo+del276-289(14bp) Homo + 294-295insCA+del270-283(14bp) Homo |
| JAK2        | 1849 G>T            | 293T-JAK2-1   | 1849G>T(COSM12600) Homo |
| KIT         | 2447 A>T            | 293T-KIT-1    | 2447A>T(COSM3134) Homo |
| KNSTRN      | 71 C>T             | 293T-KNSTRN-1 | 71C>T(COSM140056) Homo |
| KRAS        | 35 G>A              | 293T-KRAS-1   | 32insC Homo+35G>A(COSM521) Homo+del30-38(9bp) Homo |
|             |                     | 293T-KRAS-2   | 35G>A(COSM521) Homo+33delT Homo |
| Target Gene | Cell Clone Name (ID) | JCRB Cell ID | Mutation detaila |
|-------------|---------------------|--------------|------------------|
| NRAS        | 293T-NRAS-1         | 35G>A(COSM654)Homo | Homo |
|             | 293T-NRAS-2         | 35G>A(COSM654)Homo | Homo |
|             | 293T-NRAS-3         | 34G>T(COSM652)Hetero | Homo |
|             | 293T-NRAS-4         | 35G>A(COSM654)Hetero+30-31AG>GAAA Hetero | Homo |
|             | 293T-NRAS-5         | 35G>A(COSM654)Hetero+del30-44(15bp) Hetero | Homo |
| PDGFRA      | 293T-PDGFR-A-1      | 2525A>T(COSM736)Homo | Homo |
|             | 293T-PDGFR-A-2      | 2525A>T(COSM736)Hetero | Homo |
| PIK3CA      | 3140A>G             | 3140A>G(COSM775)Hetero+3143insA Hetero | Homo |
|             | 293T-PIK3CA-1       | 3144T>G(COSM27157)Homo | Homo |
|             | 293T-PIK3CA-2       | 3140A>G(COSM775)Hetero+del3131-42(12bp) Hetero | Homo |
|             | 293T-PIK3CA-3       | 3140A>G(COSM775)Hetero | Homo |
|             | 293T-PIK3CA-4       | 3140A>G(COSM775)Hetero+del3102-47(46bp) Hetero | Homo |
| PTEN        | 697 C>T             | 63-64 CG>(T,C,G)(T,C,G)(A,G) (ins 1bp for 3 alleles) | Homo |
|             | 293T-PTEN-1         | 697C>T(COSM5154)Hetero | Homo |
|             | 293T-PTEN-2         | 697C>T(COSM5154)Hetero+697-698CG>TT Hetero | Homo |
|             | 293T-PTEN-3         | 697C>T(COSM5154)Hetero+697-698CG>TT Hetero | Homo |
|             | 293T-PTEN-4         | 697C>T(COSM5154)Hetero & Del 546-672(127bp)&546 5bp ins(535-539) & Del697-715(19bp) Hetero +WT | Homo |
| SMO         | 1234C>T             | 1234C>T(COSM216037)Homo | Homo |
| STAT3       | 1919 A>T            | 1921insA Homo | Homo |

a: Mutation details include additional changes post-editing.
and targeted rate is 0.025). However, it is necessary to stay within a certain distance from the PAM site, and it is important to check in advance whether the target site is cleaved by Cas9 and gRNA, using the T7E1 assay, for example. Because we did not use any selective marker gene, we proceeded to the next step only when the cleavage was confirmed. We discontinued the target or changed the design of gRNA for those genes that did not yield clear cleaved bands in the T7E1 assay. In the case of the BRAF gene, although changing target site was effective, but we had to compromise with a relatively low mutation frequency. In the case of prioritizing the site of mutation, it is necessary to increase the efficiency of genome editing. When the “knock-in” cells are necessary, markers such as drug resistance genes can be used although that strategy is effective for a simple knockout. In such case, we propose to use the Piggy-Bac system [28], which was once utilized as the replacement for the drug resistance genes for the selection of target, and excises them with transposase to obtain the desired knock-in mutants. By using this method, we have successfully generated the RB1 mutation knock-in strain in suspension cells (Human lymphoblastoid TK6) which is generally difficult for transfection. (unpublished data).

Regarding the design of gRNA, those with few homologous sites on the genome should be selected in order to prevent nonspecific cleavage as much as possible. We have used gRNA design with one or two base mismatches where ever possible, but considerable number of three base mismatches could not be avoided [29, 30]. It is not clear how such sequence similarity affected the off-target event because we only confirmed the sequence of the target sites. It may be necessary to analyze the presence of such an off-target mutation when we characterize the phenotype of the genome-edited cells, but it is not necessary for the purpose of this study to prepare the standard cell lines (DNA) for the particular mutation. For a few genes, constant mutations other than targeted event were observed which suggest SNP in original HEK 293 T/17 strain. It should be noted that this cell line has a p53 mutation derived from large-T antigen treatment as reported [31].

The average genome-editing efficiency for knock-in mutations was around 4.5% which enabled knock-in mutant detection from 150 clones for sequencing. Regarding CRISPR/Cas9 method, Cas9 protein transfer method was also used for the later study in addition to the standard method with the Cas9 expression vector. The Cas9 protein transfer method improved the genome-editing efficiency.

It is also important to know the exact amount of the mutated alleles and their alterations during culture. It is necessary to quantitatively analyze the dosage of mutations of the standard products using the RT-PCR, the digital PCR, etc. in future.

Although the main purpose of this study is to prepare the standard reference cell lines, the created cell lines can also be used for a functional analysis of mutated genes. Since it was made with the same background of the HEK 293 T/17 cell, it is possible to compare the biological effects of introduced gene mutations with each other including hetero and homo status in some cases. Furthermore, by introducing additional gene mutations in the current strains, it can be utilized to analyze the interaction between two genes and their involvement in the process of carcinogenesis. When the NGS-based cancer gene panel tests are widely used and novel mutations with little clinical information are found, we may face a problem to distinguish whether the mutation is relevant for carcinogenesis or susceptible to certain drugs. We hope the cell-based assay such as a test for proliferation or tumorigenesis using the genome-edited cells will be developed and that will contribute additional data for the decision of clinical procedure.

Finally, the genome-edited cell lines prepared in this study can be used as a mutant standard for each target gene, which is supplied from the JCRB cell bank. These cells will also be supplied as a mixture in the future, as an all-in-one standard for cancer gene panel tests. We also hope they will be used as a standard for a cross validation between different cancer gene panels, NGS platforms, facilities or examiners.

| Target Gene | Cell Clone Name (ID) | JCRB Cell ID | Mutation detail |
|-------------|---------------------|--------------|-----------------|
| TP53        | 743 G>A             | 293T-TP53-1  | 743G>A(COSM10662)Homo+746G>A(COSM44091)Homo |
|             | 293T-TP53-2         | 743G>A(COSM10662)Hetero |
|             | 293T-TP53-3         | 743G>A(COSM10662)Homo |
|             | 293T-TP53-4         | 743G>A(COSM10662)Homo |
|             | 293T-TP53-5         | 743G>A(COSM10662)&746delG Homo |

*a& means mutations were found in the same strand, + for other cases.*

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Conclusions
In this study, we created a panel of genome edited cells for the genes frequently mutated and used in cancer gene panels such as NCC OncoPanel. These cell lines are useful for analytical validation of NGS based cancer gene panel assay. They will also be useful for a cross-platform validation of the different panels, instrument platforms, and examiners as a common standard.

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Authors' contributions
TS and AK designed the study and YT and TS performed the experiments. TS supervised the study and was a major contributor in writing the manuscript. YT, CF and MN helped design the study and provided constructive suggestions during the study. All authors read and approved the final manuscript.

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Availability of data and materials
All the representative clones isolated by genome-editing in HEK 293 T/17 cells have deposited and available at the JCRB cell bank.

Ethics approval and consent to participate
Because already established cell line (HEK 293 T/17) was used, ethics approval and consent are not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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