RESEARCH ARTICLE

A novel cell line generated using the CRISPR/Cas9 technology as universal quality control material for *KRAS* G12V mutation testing

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Background: *KRAS* mutations are the key indicator for *EGFR* monoclonal antibody-targeted therapy and acquired drug resistance, and their accurate detection is critical to the clinical decision-making of colorectal cancer. However, no proper quality control material is available for the current detection methods, particularly next-generation sequencing (NGS). The ideal quality control material for NGS needs to provide both the tumor mutation gene and the matched background genomic DNA, which is uncataloged in public databases, to accurately distinguish germline polymorphisms and somatic mutations.

Methods: We developed a novel *KRAS* G12V mutant cell line using the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) technique to make up for the deficiencies in existing quality control material and further validated the feasibility of the cell line as quality control material by amplification refractory mutation system (ARMS), Sanger sequencing, digital PCR (dPCR), and NGS.

Results: We verified that the edited cell line specifically had the G12V mutation, and the validation results presented a high consistency among the four methods of detection. The three cell lines screened contained the G12V mutation and the mutation allele fractions of G12V-1, G12V-2, and G12V-3 were 52.01%, 82.06%, and 17.29%, respectively.

Conclusion: The novel *KRAS* G12V cell line generated using the CRISPR/Cas9 gene editing system is suitable as a quality control material for all current detection methods and provides a new direction in the development of quality control material.

KEYWORDS

colorectal cancer, CRISPR/Cas9, *KRAS*, next-generation sequencing, quality control material

1 | INTRODUCTION

With the development of targeted therapy, mutations in *KRAS* have become key indicators for the effectiveness of *EGFR* monoclonal

antibody-targeted therapy and acquired drug resistance in colorectal cancer (CRC).¹⁻³ Because of the vital clinical significance of *KRAS*, the National Comprehensive Cancer Network (NCCN) highly recommends that patients with CRC be tested for the status of *KRAS* mutations

as a prerequisite for treatment modality with targeted therapy, apart from the best supportive care alone.^{1,4} Therefore, accurate detection of *KRAS* mutations is critical to clinical decision-making.

Current detection technologies for *KRAS* mutations mainly include Sanger sequencing, pyrosequencing, amplification refractory mutation system (ARMS),⁵ high-resolution melting real-time PCR (PCR-HRM),⁶ Beads, Emulsion, Amplification, and Magnetics (BEAMing),⁷ digital PCR (dPCR),⁸ and next-generation sequencing (NGS).⁹ The existing quality control materials can be broadly divided into two categories: (i) samples obtained from tumor patients, including histological samples and specific cell lines and (ii) synthetic samples, such as plasmids.¹⁰⁻¹² However, the existing quality control materials have drawbacks and do not meet the needs of all the detection methods, particularly NGS. Histological samples of patients are available in limited amounts and offer poor reproducibility because of tumor heterogeneity. Although cell lines with specific aberrations can be obtained in unlimited amounts, they do not reflect the complex composition of tumor tissues.¹¹ Even though plasmids are easy to obtain and widely used, it does not fully reflect the biological characteristics and the detection process of clinical samples due to inherent characteristics, such as the lack of a nucleic acid extraction process and background genomic DNA.

The ideal quality control material for NGS should provide both the tumor mutation gene and the matched background genomic DNA to better distinguish between single nucleotide polymorphisms (SNPs) or germline polymorphisms and somatic mutations.¹³⁻¹⁵ However, both clinical samples and specific cell lines contain SNPs from the individual itself, which are easily confused with tumor mutations. As an essential part of the standardization of clinical laboratory testing, the detection of the *KRAS* mutation urgently requires a biomimetic material that can be applied to all detection techniques. The clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) gene editing technology may offer the means to meet this requirement and provide uniform genetic background information. HEK293T cells before editing can be used as the genomic background for NGS filtration of SNPs.

In this study, using the CRISPR/Cas9 technology, we aimed to develop a *KRAS* G12V cell line that could serve as a quality control material that meets all requirements. This is the first attempt at precise genetic editing by CRISPR/Cas9 to generate quality control material. With this new perspective, the construct may be used as a control or standard in the quality control of routine clinical laboratory tests and diagnosis.

2 | METHODS AND MATERIALS

2.1 | Plasmid construction, primers, and templates design

The pSpCas9(BB)-2A-GFP plasmid (PX458; plasmid #48138) expressing Cas9 endonuclease (Addgene, Cambridge, MA) was linked with a single-guide RNA (sgRNA) designed specifically for the *KRAS* gene.¹⁶ The *KRAS* target sequence was obtained from COSMIC

(<http://cancer.sanger.ac.uk/cosmic>, last accessed 3 August 2017) and NCBI (<https://www.ncbi.nlm.nih.gov>, last accessed 3 August 2017) genetic databases. The sgRNAs were designed using the CRISPR design Web site (<http://crispr.mit.edu>, last accessed 3 August 2017). sgRNAs with a guanine (G) nucleotide append at the 5' end, higher score with the minimum off-target activity, and maximum specificity were chosen as the most appropriate. The single-stranded oligo donors (ssODNs) of sgRNAs, primers for T7 Endonuclease I (T7EI) assay, and homology-directed repair (HDR) templates (shown in Tables 1 and 2) were synthesized by Sangon Biotech (Shanghai, China).

2.2 | Cell culture and transfection

The HEK293T cell line (China Infrastructure of Cell Line Resources, Beijing, China) was chosen for experiments as it is easy to culture and displays high transfection efficiency. The cells were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific Inc., Waltham, MA) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific Inc.) at 37°C and 5% CO₂. The cells were seeded in 6-well plates at a density of 1.0×10^5 cells per well and cotransfected with the sgRNA-PX458 recombinant plasmid and HDR template during the logarithmic growth phase using Lipofectamine 3000 (Thermo Fisher Scientific Inc.), according to the manufacturer's instructions. Transfection efficiency was assessed by the expression of green fluorescent protein (GFP). After transfection for 48 hours, the cells were harvested and DNA was extracted using the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany). Single cells with high fluorescence were then sorted by flow cytometry (BD FACSAria II; BD Biosciences, Franklin Lakes, NJ) within a week.

2.3 | Validation of mixed clonal cells

The Eppendorf BioPhotometer was used to quantify DNA concentration. The cleavage efficiencies of sgRNAs were evaluated by the T7EI assay using T7 Endonuclease I (New England BioLabs Inc., Ipswich, MA).¹⁶ The PCR system contained 25 µL PrimeSTAR Max DNA Polymerase (Takara Bio Inc., Shiga, Japan), 1.5 µL T7EI forward and reverse primers ($10 \mu \text{mol L}^{-1}$), and 200 ng DNA and was made up to 50 µL with nuclease-free water. The amplification conditions were as follows: 98°C for 3 minutes; followed by 35 cycles at 98°C for 10 seconds, 53°C for 5 seconds, and 72°C for 35 seconds; and 72°C for 2 minutes. Following amplification, the

TABLE 1 The sequences of sgRNAs and primers

Name	Sequences(5'→3')
sgRNA1	CTGAATTAGCTGTATCGTCA
sgRNA2	GAATATAAAGTTGTGGTAGT
T7EI-F	TTTAGCCGCCGAGAACA
T7EI-R	AGAATGGTCCTGCACCAAGTAAT
PX458 U6-F	GAGGGCTATTTCCCATGATTC

TABLE 2 The sequences of HDR templates

Name	Sequences(5'→3')
G12D-HDR template	TTCATTATTTTATTATAAGGCCTGCTGAAAATGACTGAATATAAACTTGTGGTAGTTGGAGCTGATGGCGTAGGCAAG AGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACGAATATGAT
G12V-HDR template	TTCATTATTTTATTATAAGGCCTGCTGAAAATGACTGAATATAAACTTGTGGTAGTTGGAGCTGTTGGCGTAGGCAAG AGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACGAATATGAT
G13D-HDR template	ATTATTTTATTATAAGGCCTGCTGAAAATGACTGAATATAAACTTGTGGTAGTTGGAGCTGGTGACGTAGGCAAGAGT GCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACGAATATGATCA

cleavage efficiencies were analyzed according to the T7EI manufacturer's instructions. After quantification using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific Inc.), the mixed clone DNA was diluted to 0.5–1 ng/μL and the human *KRAS* gene mutation detection kit (AmoyDx, Xiamen, China) was used to detect the mixed clones on an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. The results were analyzed using SDS software, version 1.3.0 (Applied Biosystems).

2.4 | Detection of the mutation in the monoclonal cells using Sanger sequencing and ARMS PCR

DNA was extracted from monoclonal cells using the QIAmp DNA Mini Kit (Qiagen) and quantified by spectrophotometry. The PCR products from the genomic DNA of all monoclonal cells were directly sequenced. The clones that screened positive using Sanger sequencing were verified by ARMS PCR, as described above. After verification (Figure 1), part of the mixed clonal cells and the positive monoclonal cells were stored in liquid nitrogen. In addition,

by ARMS, we evaluated the stability of the mutant clones during passaging.

2.5 | Detection of the mutation using the QuantStudio 3D Digital PCR System

The QuantStudio 3D dPCR System (Thermo Fisher Scientific Inc.) is an accurate mutation allele fraction (MAF) detection method and was used to validate the generated cell line. Probes specific for G12V were used for the current analysis; the corresponding assay was Hs000000050_rm (c.35G>T). Reactions were performed in a final volume of 15 μL containing QuantStudio 3D Master Mix (7.5 μL), primer/probe mixture (0.75 μL), and diluted DNA (6.75 μL). The reaction mixture was loaded onto a QuantStudio 3D Digital PCR 20K Chip (Thermo Fisher Scientific Inc.) using a chip loader. Thermal cycling was performed on the ABI 9700 (Applied Biosystems), according to the manufacturer's instructions. The dPCR chip was read using the QuantStudio 3D Chip Reader (Thermo Fisher Scientific Inc.), and the data were analyzed using the QuantStudio 3D AnalysisSuite (Version 2.0.0-Rel).

FIGURE 1 Schematic and experimental flow diagram of the CRISPR/Cas9 gene editing technology

2.6 | Detection of the mutation using NGS

The sequencing library was prepared from 10 ng DNA using the Ion Torrent Ampliseq kit 2.0-96 LV (Thermo Fisher Scientific Inc.), following the manufacturer's instructions. The DNA was bar-coded using the Ion Xpress Barcode Adapters 1-96 Kit and quantified by quantitative PCR (qPCR), using the Ion Library TaqMan[®] Quantitation Kit (Thermo Fisher Scientific Inc.) after purification of libraries. Emulsion PCR was performed using the Ion PGM Hi-Q View OT2 Kit and the Ion OneTouch 2 system (Thermo Fisher Scientific Inc.), according to the manufacturer's instructions. Template-positive ion sphere particles (ISPs) were enriched using the Ion Torrent OneTouch ES (Thermo Fisher Scientific Inc.). Enriched ISPs were loaded on a PGM 318 chip and sequenced using the Ion PGM Hi-Q View Sequencing Reagents.

3 | RESULTS

3.1 | Generation of KRAS G12V cells using the CRISPR/Cas9 system

The CRISPR/Cas9 system was used to edit the genome of HEK293T cells to generate KRAS mutation hotspots. HDR templates of the three most common mutation types, KRAS G12D/G12V/G13D, were added to the transfection system. To obtain the best possible transfection efficiency, 500 ng and 1 μ L were taken as the optimum amounts of the PX458-sgRNA recombinant plasmid and the HDR template (10 μ mol L⁻¹), respectively. The transfection efficiency of the recombinant plasmid was about 70%. The T7EI electrophoresis results showed that the cleavage efficiency of sgRNA2 was higher than that of sgRNA1 (Figure 2). Next, ARMS PCR was used to verify that the CRISPR/Cas9-edited cells specifically contained the KRAS G12V mutation (Figure 3A). We performed flow cytometry and sorted single cells with a high GFP fluorescence signal to isolate positive monoclonal cells.

3.2 | Detection of the mutation using ARMS PCR, Sanger sequencing, QuantStudio 3D Digital PCR System, and NGS

The ARMS PCR and Sanger sequencing results are shown in Figure 3. We directly sequenced the PCR products of DNA isolated from the cells. The positive cells were identified with the specific mutation c.35G>T using Sanger sequencing. From the results of the sequence peaks, we observed the presence of wild-type genes at the mutation site (Figure 3D). We then linked the PCR products with the pMD18-T vector (Takara Bio Inc.) and sequenced the ssDNA (Figure 3E). Except for mutant T and wild-type G, the sequencing products did not contain any other unrelated genes. We screened out only positive monoclonal cells with the G12V mutation. Next, ARMS PCR was used to validate the mutation. The amplification curves of the G12V-positive edited cell lines and the SW480 cell line are shown in Figure 3B. There were no significant differences between the results of the positive cell lines and the SW480 cell line ($P > .05$, Figure 3C). After cell passage, there

Mutant type	+	+	-	+	+	-
Wild type	-	+	+	-	+	+
T7EI	+	+	+	+	+	-

FIGURE 2 T7EI assay for the cleavage efficiency of sgRNA1/2. Lanes 1, 5, and 9 show the DNA marker of 2000 bp, lanes 2-4 show the cleavage efficiency of sgRNA1, and lanes 6-8 show the cleavage efficiency of sgRNA2

were no statistical differences between the first and fifth generations of the monoclonal cell lines ($P > .05$, Figure 3C). Therefore, our novel cell lines were stable through passaging.

3.3 | Detection of the mutation using the QuantStudio 3D Digital PCR System and NGS

To further validate the feasibility of the quality control material, we generated and calculated the MAFs of the genomic DNA from the cells; we performed dPCR with the QuantStudio 3D dPCR System and NGS using the Ion PGM platform. We observed that all three cell lines contained the G12V mutation and the MAFs of G12V-1, G12V-2, and G12V-3 were 52.01%, 82.06%, and 17.29%, respectively (Figure 4). Results from targeted sequencing revealed only the presence of the KRAS G12V mutation in the three positive cell lines as well as in SW480. The MAFs in the edited cell lines derived from NGS were in agreement with the dPCR results. The SW480 and HEK293T cell lines were used as the positive and negative control, respectively, in all the methods. Figure 5 shows the specific mutation present in the three positive cell lines.

4 | DISCUSSION

Presenting a more aggressive phenotype among the most common KRAS mutations, G12V is associated with worse cancer progression and poorer clinical outcome compared with CRC with wild-type KRAS.¹⁷⁻²⁰ The accurate detection of the mutation in clinical

laboratories continues to be an integral part of precision medicine. Owing to the demand for large amounts of quality control material, it becomes impractical to provide the same material to all laboratories, especially with the use of surgical pathological material.²¹ As

the first successful attempt at precise gene editing to produce universal quality control material using the CRISPR/Cas9 system, our study shows that this idea is feasible and promising. With features of easy access and full simulation of clinical samples, the generated

FIGURE 5 The NGS results for the edited and the SW480 cell lines. The mutation was identified in the edited cell lines with the corresponding MAFs

cell line indicates a shift in the direction of development of quality control material and may better contribute to the standardization of clinical laboratory testing.

Primarily, this quality control material can be used for various detection methods. Our results show that the genome of the results for edited KRAS G12V cell line and the SW480 cell line were 100% consistent for all methods. Moreover, the cell line generated from HEK293T cells can provide specific information about SNPs and germline polymorphisms that cannot be found in public databases¹³⁻¹⁵ and is hence more suitable for NGS than other material.

Additionally, compared to synthetic or spike-in samples, the edited cells can be mixed with HEK293T cells at any precise ratio to provide a quantitative assessment of the MAFs. For example, the genomic DNA of the cell line can be mixed with the genomic DNA of HEK293T cells with the same genetic background and digested to yield cell-free DNA (cfDNA). Our team has recently generated synthetic cfDNA with somatic mutations as quality control material for NGS using CRISPR/Cas9-edited HEK293T cells and site-directed mutagenesis of DNA fragments that have a genetic background identical to that of HEK293T cells.²² Micrococcal nuclease (MNase)-digested DNA showed an enrichment of 147 bp and has been proven to completely simulate the biological properties of the clinical plasma cfDNA samples.²²

Furthermore, a subcutaneous xenograft model can be prepared by injecting the cell suspension into flanks of nude mice. The xenografted tumors can then be used to produce formalin-fixed, paraffin-embedded (FFPE) samples, which have typical histological structures similar to those of tumors from patients and can fully simulate pathological tissue samples. The *EML4-ALK* rearrangement FFPE samples based on CRISPR/Cas9 editing and xenografts have proved to be applicable as candidate reference materials.²³ From this point of view, the CRISPR/Cas9 gene editing technique can be used in the field of quality control, in addition to its use in treatment and molecular research.

In conclusion, our work introduces a novel and universal cell line with the *KRAS* G12V mutation, produced using the CRISPR/Cas9 system, that can help make the detection process amenable to monitoring and has the potential to meet the current requirements

for controls or standards for all current detection methods. This technology provides a new direction in the study of quality control materials for the standardization of clinical laboratory tests. With identical genetic background and by passing the verification by various tests, this technology is suitable for all of the existing *KRAS* G12V detection methods.

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