Exploration of genetic basis underlying individual differences in radiosensitivity within human populations using genome editing technology

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ABSTRACT

DNA double-strand breaks (DSBs) induced by ionizing radiation (IR) are the initial and critical step in major alteration of genetic information and cell death. To prevent deleterious effects, DNA repair systems recognize and re-join DNA DSBs in human cells. It has been suggested that there are individual differences in radiosensitivity within human populations, and that variations in DNA repair genes might contribute to this heterogeneity. Because confounding factors, including age, gender, smoking, and diverse genetic backgrounds within human populations, also influence the cellular radiosensitivity, to accurately measure the effect of candidate genetic variations on radiosensitivity, it is necessary to use human cultured cells with a uniform genetic background. However, a reverse genetics approach in human cultured cells is difficult because of their low level of homologous recombination. Engineered endonucleases used in genome editing technology, however, can enable the local activation of DNA repair pathways at the human genome target site to efficiently introduce genetic variations of interest into human cultured cells. Recently, we used this technology to demonstrate that heterozygous mutations of the ATM gene, which is responsible for a hyper-radiosensitive genetic disorder, ataxia-telangiectasia, increased the number of chromosomal aberrations after IR. Thus, understanding the heterozygous mutations of radiosensitive disorders should shed light on the genetic basis underlying individual differences in radiosensitivity within human populations.

Keywords: genome editing technology; individual differences in radiosensitivity; hyper-radiosensitive disorders

INTRODUCTION

Ionizing radiation (IR) induces DNA double-strand breaks (DSBs), causing genetic instability and cell death, through which acute radiation syndrome (ARS) and/or carcinogenesis can occur. In human cells, DNA repair systems recognize and repair DNA DSBs to prevent IR-induced clinical symptoms [1]. The term ‘radiosensitivity’ refers to various phenomena and is defined by the biological steps. Conventionally, radiosensitivity has been quantified using the level of mortality of cells upon exposure to IR, which is the main factor contributing to the incidence of acute IR-induced tissue damage. To analyze the tendency for IR-induced carcinogenesis to develop, cellular radiosensitivity should be measured by determining the chromosomal aberrations after IR, which is a technique for monitoring the capacity for DNA DSB repair. Based on a series of studies associated with chromosomal instability after IR, it has been postulated that there are individual differences in the capacity to perform DNA DSB repair within human populations [2, 3], which we define as ‘radiosensitivity’ in this review. Variation in the genes involved in DNA repair might underlie the
individual differences in radiosensitivity. To shed light on this issue, it is informative to count the chromosomal aberrations after IR in primary cells, such as skin fibroblasts and peripheral lymphocytes, with a sequence variation of the susceptible gene. However, the radiosensitivity in primary cells might be affected by confounding factors including smoking and the diverse genetic backgrounds within human populations [4]. It is therefore important to quantify the effect of a candidate nucleotide variation on radiosensitivity in a human cultured cell line with a uniform genetic background.

Gene targeting in human cultured cells is generally difficult because of their limited level of homologous recombination. Engineered endonucleases (EENs), including zinc finger nucleases (ZFNs), transcription activator-like nucleases (TALENs), and clustered regulatory interspaced short palindromic repeat/Cas9-based RNA-guided DNA endonucleases (CRISPR/Cas9) recognize and introduce a DSB at a target DNA sequence, thereby increasing the efficacy of genome editing through the site-specific activation of DNA repair activity [5–7]. Genome editing technology enables the introduction of candidate nucleotide variation underlying radiosensitivity into human cultured cells with a uniform genetic background. Comparison of chromosome aberrations after IR in such edited cells can then clarify whether a nucleotide variation of interest is indeed involved in radiosensitivity [8]. In this review, we discuss the application of genome editing technology in human cultured cells to studies on the genetic basis underlying individual differences in radiosensitivity.

GENETIC FACTORS UNDERLIE INDIVIDUAL DIFFERENCES IN RADIOSENSITIVITY WITHIN HUMAN POPULATIONS

The cellular capacity for DNA DSB repair underlying radiosensitivity can be quantified using several established assays. The cytokinesis-blocked micronucleus (CBMN) assay monitors cellular radiosensitivity by counting micronuclei derived from unrepared DSB-induced chromosomal fragments [9]. This assay has demonstrated that compared with a small population of healthy individuals, breast cancer patients showed mildly attenuated DNA DSB repair after IR [2], implying that there are indeed individual differences in radiosensitivity within human populations. The fluorescent in situ hybridization (FISH) painting assay, which detects IR-induced unstable chromosomal structural abnormalities such as ring and multicentric chromosomes, also supported the existence of this heterogeneity [10]. Importantly, a number of genome-wide association studies (GWASs) have also shown that many nucleotide variations in the genes associated with DNA repair cosegregate with an increased risk of cancer in normal individuals [11], suggesting that these genetic variations determine the individual capacity for DNA DSB repair after IR. Interestingly, other nucleotide variations in inflammation-related genes (ILs, TNF and TGF-β1), stress response–related genes (MTHFR and HSPB1), and angiogenesis–related genes (VEGF) have also been shown to be epidemiologically associated with the toxicities of radiation therapy [12, 13]. However, further studies are needed to dissect how these variations in non-DNA repair genes contribute to cellular radiosensitivity mechanistically.

In general, nucleotide variations with a low frequency in the population have large effects on the biological process [14]. In fact, most hereditary hyper-radiosensitive disorders are due to rare mutations in DNA repair genes of large effect [15, 16]. The best-known example is a rare autosomal-recessive disease, ataxia-telangiectasia (A-T; Online Mendelian Inheritance in Man (OMIM): 067585), which is characterized by cancer predisposition, infertility, immunodeficiency, and neurodegeneration. Germline mutations in the ataxia-telangiectasia mutated (ATM) gene encoding ATM kinase, which is a DNA DSB damage response master kinase, cause A-T at a frequency of 1/10 000 to 1/300 000 [15, 17, 18]. The cells from A-T patients show remarkable chromosomal aberrations and lethality after IR. A-T heterozygous carriers are clinically asymptomatic and constitute ~1% of human populations [15, 18]. Interestingly, it has been reported that they exhibit a significantly increased risk of breast and ovarian cancers compared with non-carriers [19]. These findings have led us to clarify whether A-T heterozygous carriers are more radiosensitive than normal individuals. Previous studies demonstrated that the fibroblasts and peripheral lymphocytes from A-T heterozygous carriers have increased chromosomal structural abnormalities after IR [20], while it was also shown that the radiosensitivities of the carriers and normal individuals were not clearly segregated because of the heterogeneous genetic backgrounds in some cases [21]. To ensure that reliable results regarding the cellular radiosensitivity of A-T heterozygous carriers were obtained, we used the automatic Metafer system to detect micronuclei in cytokinesis-blocked binucleated cells after IR. The automatically obtained images of >1000 binucleated cells were visually evaluated (i.e. a semiautomatic approach) to remove pseudo-positive and/or negative micronuclei and binucleated cells. The semiautomatic CBMN assay revealed that the skin fibroblasts from A-T heterozygous carriers formed IR-induced micronuclei more frequently than those from normal individuals [22]. Consistent with this, chromosomal aberration analysis using peptide nucleic acid (PNA)-FISH probes, which enable the rapid and clear staining of telomeres and centromeres, demonstrated mild radiosensitivity in the cells from A-T heterozygous carriers [22]. Notably, both the semiautomatic CBMN assay and the chromosomal aberration analysis also detected the individual differences in IR-induced micronucleus formation within A-T heterozygous carriers and within normal individuals [22]. These results suggest that the copy number of ATM mutations increases cellular radiosensitivity, and that it is difficult to precisely quantify the effect of ATM mutations on cellular radiosensitivity in the presence of the diverse genetic backgrounds harbored by individuals in a human population.

CRISPR/OBLIGARE-MEDIATED GENE TARGETING ENABLES EVALUATION OF THE EFFECT OF HETEROZYGOUS MUTATIONS IN THE DNA REPAIR GENES ON CELLULAR RADIosensitivity in a uniform genetic background

Genome editing technology in human cultured cells with a uniform genetic background might be a direct approach for measuring the effect of nucleotide variation on cellular radiosensitivity. DNA DSBs at the target sequence induced by EENs are repaired mainly by error-prone non-homologous end joining (NHEJ) or error-free homologous directed repair (HDR) [23, 24]. NHEJ, which is active throughout the cell cycle, generates insertions or deletions (indels) of various lengths that can cause frameshift mutations and, consequently, gene knockout [23]. In contrast, HDR, which occurs in late-S and G2 phases, leads to a precise recombination event between a homologous DNA donor and the
mildly radiosensitive phenotype in comparison with resistant clones. Semiautomatic CBMN assay and chromosomal aberrations of the A-T carrier model of A-T were isolated at a rate of 3.3% of the drug-resistant clones. In contrast, monoallelic inserted clones were dominant (at ~0.05%) and the ends of the transgene contain uncontrolled indels.

Currently, genome editing technology is mainly applied to human cancer cell lines, such as HEK293T cells and HCT116 cells, which have intrinsic HDR activity that is sufficient for isolating genome-edited cell clones efficiently. Since it was shown that IR-induced cellular responses in normal tissues were significantly different from those in cancer tissues [25], genome editing technology in a normal-tissue-derived cell line should be more optimal for an experimental system evaluating cellular radiosensitivity. However, HDR-dependent genome editing is limited in normal-tissue-derived cell lines because of their inefficient HDR activity. For instance, we previously generated a microcephaly-associated KIF2A gene knockout hTERT-RPE1 cell line, which is derived from normal retinal tissue, using TALEN and a drug-resistant gene cassette contained in a homology arm-tagged targeting vector, but the efficacy of isolation of the HDR-mediated knockout clones was low, at ~1% of drug-resistant clones [26]. In contrast, Maresca et al. inserted a ZFN site located in the genome into a drug-resistant gene cassette vector without long-homology arms, and introduced the ZFN and the targeting vector into human cultured cells to generate the targeted clones with high efficacy through NHEJ activity [27] (Fig. 1). They named this method ObLiGaRe (obligate ligation-gated recombination), based on the Latin verb obligare (‘to join to’) [27]. The ObLiGaRe method enables enhancing of the efficiency of the targeted insertion ~50 times more than the conventional HDR-mediated targeting with EENs in human cultured cells, but the orientation of the targeting donor DNA is not controlled (Fig. 1). To model A-T heterozygous carriers in hTERT-RPE1 cells, we combined CRISPR/Cas9 with the ObLiGaRe approach [22]. In this method, biallelic targeting vector-inserted clones corresponding to ATM-knockout (ATM−/−) cells were a minor component (at ~5%) among the drug-resistant clones, while the monoallelic inserted clones were dominant (at >70%). Because 95.4% of monoallelic inserted clones carried the NHEJ-mediated indels at the target locus in the second, uninserted allele, >70% of the drug-resistant clones were indeed ATM−/− cell clones. In contrast, ATM heterozygous knockout (ATM+/-) cells as a carrier model of A-T were isolated at a rate of 3.3% of the drug-resistant clones. Semiautomatic CBMN assay and chromosomal aberration analysis demonstrated that ATM+/- cell clones exhibited a mildly radiosensitive phenotype in comparison with ATM+/+ cell lines, suggesting that ATM heterozygous mutations were indeed a genetic factor underlying the heterogeneity of radiosensitivity. Importantly, the differences in radiosensitivity within the clones with the same genotype after genome editing were small, unlike the individual differences in primary cells from the A-T heterozygous carriers. The CRISPR/ObLiGaRe method is thus a powerful tool for evaluating the effect of mutations in DNA repair genes on radiosensitivity.

Besides A-T, a number of hereditary disorders caused by germline mutations of the DNA repair genes have been reported, such as A-T-like disorders (MRE11A) [28], Nijmegen breakage syndrome (NBS; NBS1) [29], NBS-like disorder (RAD50) [30], Riddle syndrome (RNFI168) [31], radiosensitive severe combined immunodeficiency (Artemis) [32], Lig-IV syndrome (DNA Lig IV) [33], and microcephalic primordial dwarfism (XRCC4) [34] (Table 1). Moreover, heterozygous BRCA1 and BRCA2 mutations cause hereditary breast and ovarian cancers (HBOCs) [35]. The populations of patients with these hereditary disorders are generally small, while both heterozygous carriers and BRCA1- or BRCA2-mutated HBOC patients are estimated to constitute ~0.05%–1% of the general population [36, 37]. Whether these heterozygous mutations contribute to the cellular radiosensitivity is a key question for exploring the genetic basis underlying the individual differences in radiosensitivity within human populations [38], these mutations are potential targets for application of the CRISPR/ObLiGaRe method.

**THE EEN-MEDIATED SINGLE-BASE-PAIR EDITING TECHNIQUE MIGHT ENABLE DIRECT EVALUATION OF SINGLE-NUCLEOTIDE VARIATION (SNV) UNDERLYING CELLULAR RADIOSensitivity**

To confirm whether SNVs in the DNA repair genes related to various cancer risks influence the cellular radiosensitivity directly, cointroduction of EENs and 100-200-mer single-stranded DNA oligonucleotides (ssODNs) with the SNV into human cultured cells has recently been introduced and become established [39, 40]. Using this method, we recently generated HCT116 cell clones with knock-in of the primary
| Disease                                      | Causative gene | OMIM   | Genetics | Impaired DNA damage response | Clinical Features | Frequency of patients | Frequency of carriers | References |
|---------------------------------------------|----------------|--------|----------|------------------------------|-------------------|-----------------------|-----------------------|------------|
| Ataxia telangiectasia (A-T)                 | ATM            | 607585 | AR       | HR                           | Neurodegeneration | +                     | −                     | 1/40,000 - 1/300,000 | 0.36-1%    |
| A-T–like disorder (A-TLD)                   | MRE11A         | 604391 | AR       | HR, NHEJ                     | Neurodegeneration | +                     | −                     | <1/100,000  | 0.2-0.5%   |
| Nijmegen breakage syndrome (NBS)           | NBS1           | 251260 | AR       | HR, NHEJ                     | Microcephaly     | +                     | −                     | <1/200,000  | 0.05-1%    |
| NBS-like disorder (NBSLD)                   | RAD50          | 613078 | AR       | HR, NHEJ                     | Microcephaly     | −                     | −                     | 2 cases described | Not estimated |
| Riddle syndrome                             | RNF168         | 611943 | AR       | HR, NHEJ                     |                   | +                     | −                     | 2 cases described | Not estimated |
| Radiosensitive severe combined immunodeficiency (RS-SCID) | Artemis | 602450 | AR       | NHEJ                         |                   | +                     | −                     | 34 cases described | Not estimated |
| LigIV syndrome                              | DNA LigIV      | 606593 | AR       | NHEJ                         | Microcephaly     | +                     | −                     | 11 cases described | Not estimated |
| Microcephalic primordial dwarfism (MPD)     | XRCC4          | 616541 | AR       | NHEJ                         | Microcephaly     | +                     | −                     | 5 cases described | Not estimated |
| Hereditary Breast and Ovarian Cancer syndrome (HBOC) | BRCA1       | 604370 | AD       | HR                           |                   | −                     | −                     | 1/300       | 0.13-0.2%  |
|                                             | BRCA2          | 600185 | AD       | HR                           |                   | −                     | −                     | 1/800       | <0.13%     |

AR = autosomal recessive, AD = autosomal dominant, HR = homologous recombination, NHEJ = nonhomologous recombination.
microcephaly-causing missense mutation of the WDR62/MCPH2 gene to demonstrate that this mutation affected the spindle orientation underlying symmetric cell division \[41\]. However, it is difficult to isolate SNV-knock-in cell clones because this method is dependent on HDR activity.

To improve the efficacy of generation of SNV-knock-in cell clones, we previously developed a TALEN-mediated two-step single-base-pair editing strategy \[42\] (Fig. 2). The first step is TALEN-mediated insertion of a drug-selectable marker cassette into a target flanking region with the SNV (asterisk in Fig. 2). The targeting vector also contains a neomycin-resistance gene and a herpes simplex virus thymidine kinase (hsvTK) gene separated by a 2A peptide sequence, expressing the discrete protein products from a single open reading frame. Neomycin-mediated positive selection enables isolation of the drug-selectable marker cassette knock-in cell clones. The second step is removal of the drug-selectable marker cassette from the targeted alleles using TALENs, and simultaneous introduction of the SNV-carrying DNA template in an HDR-activity-dependent manner. When HDR-mediated insertion of the hsvTK-2A-Neo cassette occurs, the DSB-target site for the EEN used at the first step should be excised out. Thus, it is necessary to construct the EENs specific for removal of the cassette from the target (Fig. 2). The SNV-knock-in clones were negatively selected using ganciclovir treatment. Using this technique, we identified a causal mutation of a cancer-prone genetic disorder, premature chromatid separation with mosaic variegated aneuploidy [PCS (MVA)] syndrome \[42\]. This is a rare autosomal recessive disease characterized by constitutional numerical chromosomal abnormality, a high risk of childhood cancers, and the typical spectrum of ciliopathies such as polycystic kidney and Dandy-Walker anomaly \[43\]–\[45\]. Both biallelic and monoallelic mutations of the BUB1B gene encoding a central player in the mitotic spindle assembly checkpoint, BubR1, have been reported in patients with PCS (MVA) syndrome \[46\],\[47\]. Monoallelic mutations in the exons of BUB1B were identified in seven Japanese families with this syndrome. No second mutation in exons of the BUB1B gene was detected in the opposite allele, although a conserved BUB1B haplotype within a 200-kb interval linked to the reduced transcript level among the Japanese patients was identified \[47\]. Deep-sequencing analysis of this haplotype revealed that a unique SNV in an intergenic region 44 kb upstream of the BUB1B transcription start site cosegregated with the disease \[42\]. We used TALEN-mediated single-base-pair editing technology to efficiently generate SNV-knock-in HCT116 cell clones.

Fig. 2. The EEN-mediated two-step single-base-pair editing strategy for scarless SNV knock-in. In the first step of this strategy, a drug-selection cassette is introduced into the target locus along with the SNV(asterisk). In the second step, the targeted cassette is excised and single-base substitution is simultaneously introduced.
genome-edited clones exhibited reduced BUB1B transcript levels and increased chromosomal instability, demonstrating that this SNV was indeed the causal mutation of PCS (MVA) syndrome [42]. The single-base-pair editing technique is thus useful for investigating nucleotide variants with unknown functional relevance.

With this approach, TALEN can be replaced by CRISPR/Cas9. The EEN-mediated drug-selectable-SNV knock-in technique can introduce a single-nucleotide substitution anywhere within the genome, without any footprints besides targeted sites in human cultured cells, but this requires at least two rounds of clone selection, which is time-consuming and can produce off-target mutations. Further technical improvements are thus needed to establish efficient scarless SNV-knock-in systems.

CONCLUSION

Genome editing technology in human cultured cells has provided new insights into the genetic basis underlying individual differences in radiosensitivity within human populations. In future, the nucleotide variations linked to radiosensitivity validated by a reverse genetics approach might contribute to generating a personal radiation protection standard for practical and clinical situations, such as disasters involving the release of a large amount of radiation, radiation therapy, and CT imaging. Genome editing technology experimentally demonstrated that ATM heterozygous mutations indeed determine individual differences in radiosensitivity [22]. In the field of radiation biology, it is still unresolved whether heterozygous mutations in the genes causative of other hyper-radiosensitive diseases are also involved in this heterogeneity. In addition, SNVs epidemiologically associated with the toxicities of radiation therapy and cancer risks are also potential targets for reverse genetics studies of individual differences in radiosensitivity. However, the SNV knock-in techniques are still limited because of the general characteristic of human cultured cells that their DNA DSBs are predominantly re-joined by NHEJ rather than by HR. Therefore, further development of HR-independent SNV-knock-in methods [48, 49] and small compounds for controlling the balance between NHEJ and HR [50–53] are required in order to understand the genetic determinants of individual differences in radiosensitivity using genome editing technology. Interestingly, it has been reported that several cytidine deaminases, including APOBEC1 and AID fused to catalytically dead Cas9(dCas9), enabled the conversion of C · G to T · A at the specific base pair [54, 55]. In addition, a recent report also demonstrated that a chemically evolved tRNA adenosine deaminase fused to dCas9 converted target A · T to G · C in genomic DNA [56]. These technical advances in genome editing technology might provide new insights into the genetic basis underlying radiosensitivity within human populations.

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