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Application of Host Cell Reactivation in Evaluating the Effects of Anticancer Drugs and Environmental Toxicants on Cellular DNA Repair Activity in Head and Neck Cancer

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1. Introduction

DNA repair pathways are targets of numerous anticancer drugs including natural and chemical compounds, which direct cancer cells toward apoptosis. However, different types of cancer cells consist of various alterations in DNA repair genes that make cancer cells become drug-resistant and lead to treatment failure and disease recurrence. On the contrary, cancer cells may also possess defects in certain DNA repair pathway that make them are susceptible to certain compounds, which inhibit another DNA repair pathway inside the cancer cells. As a result, these compounds selectively kill the cancer cells and are less harmful to the normal ones. Understanding the effects of anticancer drugs on DNA repair as well as the DNA repair activity of cancer cells themselves are important for improvement of anticancer treatment. Similarly, this information is helpful for elucidation of the carcinogenicity of environmental toxicants. This chapter introduces the crosstalk between anticancer drugs, environmental toxicants and DNA repair pathways in head and neck cancer. In addition, the application of an easy, fast and measurable *in vivo* functional assay for nucleotide excision repair (NER) and DNA repair via homologous recombination (HR) and non-homologous end-joining (NHEJ) pathways is shown to examine the cellular DNA repair activity responding to anticancer drugs or environmental toxicants. By which the functional roles of DNA repair genes in response to anticancer treatments and genotoxic substances could be evolved in head and neck cancer cells.

2. Roles of DNA repair genes/pathways in cancer development and treatment

DNA repair genes play a pivotal role in the maintenance of genome integrity. Defects or dysregulation of DNA repair genes can result in genomic instability (GIN), which is a common feature of cancer cells (Hanahan & Weinberg, 2000). To prevent this, human cells evolve several

DNA repair pathways that may interplay each other to repair various types of DNA damages. These DNA repair mechanisms include pathways of base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), double strand break (DSB) repair through homologous recombination (HR) or non-homologous end-joining (NHEJ) and direct repair of DNA lesions such as O^6-methylguanine by O^6-methylguanine-DNA methyltransferase (Sancar et al., 2004). Collectively, there are hundreds of DNA repair genes involved in various DNA repair pathways that include processes of sense and recognition of DNA lesions, amplification and transmission of the damage signal, recruitment of repair proteins to the damage sites, and execution of DNA repair (Sancar et al., 2004; Wood et al., 2005).

2.1 DNA repair genes/signaling in HR and NER pathways

Upon DNA damaged, histone H2AX, a histone H2A variant, is quickly phosphorylated (denoted as γ-H2AX) in an ataxia telangiectasia mutated (*ATM*)-dependent manner (Uziel et al., 2003). The phosphorylated H2AX serves as an important marker for DNA damages. Some genes are also involved in the recognition of DNA damages. They are members of MRN complex (*Mre11A, RAD50, NBN*) for DSB and damage-specific DNA binding protein 1 and 2 (*DDB1* and *DDB2*), Xeroderma pigmentosum (XP) complementation group C (*XPC*) for UV-induced damages and bulky DNA adducts, which are produced by DNA-damaging chemotherapeutic drugs and can be repaired through NER pathway. In addition to H2AX, ATM also phosphorylates p53, BRCA1, CHEK1/2 and results in activation of various DNA repair pathways as well as induction of cell cycle arrest (Sancar et al., 2004). Generally, the genes involved in HR repair include *BRCA1, BRCA2*, members of *RAD51* and Fanconi anemia (FA) families, as well as the Bloom syndrome, RecQ helicase-like (*BLM*) and Werner syndrome, RecQ helicase-like (*WRN*). The genes in NER pathway consists of XP complementation group A to G (*XPA* to *XPG*), XP complementation group variant (*XPV*), excision repair cross-complementing rodent repair deficiency, complementation group 1 (*ERCC1*), replication protein A (*RPA*), and so on (Sancar et al., 2004; Wood et al., 2005).

ATM is the key gene for initiating DNA repair signaling. Its downstream targets, both *TP53* and *BRCA1* are capable of regulating multiple DNA repair pathways (Deng, 2006; Helton & Chen, 2007). *BRCA1* encodes a multifunctional protein that maintains genome integrity through regulating gene transcription, cell cycle checkpoints, DNA repair (Deng, 2006; Yoshida & Miki, 2004), and centrosome duplication (Deng, 2002; Xu et al., 1999). In addition to the role in HR, BRCA1 is involved in NER through transactivating the expression of *DDB2* and *XPC* (Hartman & Ford, 2002; Takimoto et al., 2002), both of them can also be transactivated by p53 (Adimoolam & Ford, 2002; Hwang et al., 1999). Thus, both *BRCA1* and *TP53* can regulate NER pathway. RAD51 is the human homolog of bacteria *recA* and forms a complex with BRCA1 and BRCA2. This interaction is important for proper regulation of RAD51 activity inside a cell. Loss of the binding between RAD51 and BRCA complex may be a key event leading to GIN and tumorigenesis (Martin et al., 2007). RAD51 contributes the key step of HR by mediating homologous pairing and strand exchange between two homologous DNA (Richardson, 2005). It has been shown that *RAD51* overexpression is correlated with GIN and that p53 can transcriptionally inhibits *RAD51* expression (Arias-Lopez et al., 2006; Richardson et al., 2004).

2.2 DNA repair activity is important for preventing cancer development

Activation of DNA repair genes has been shown as a critical anticancer barrier in early human tumorigenesis. By examining various stages from precancerous lesions to late-stage

tumor tissues, DNA repair genes, including *ATM, CHEK1/2*, and *TP53*, are found predominantly to be highly activated in the precancerous stage of bladder, colon and lung epithelia when DNA damages are emerging inside these cells (Bartkova et al., 2005; Gorgoulis et al., 2005; Venkitaraman, 2005). Further, DNA repair genes also play a key role in the oncogene-induced senescence and prevent cell transformation (Bartkova et al., 2006; Braig et al., 2005; Di Micco et al., 2006). In other word, cells that are unable to activate DNA repair genes in the early-stage of tumorigenesis are susceptible for malignant transformation. These data demonstrated in somatic cancers strongly indicate that defects or inactivations of DNA repair genes/pathways are prerequisite for tumor development. Besides, several cancer predisposition syndromes are linked to hereditary mutations or deletions of DNA repair genes, such as *ATM* in ataxia telangiectasia, *BRCA1* and *BRCA2* in familial breast and ovarian cancers, *XPC* and *DDB2* in Xeroderma pigmentosum. Hence, people with, either inherited or sporadic, inactivated DNA repair genes/pathways are prone to cancer development. In this chapter, we will use head and neck cancer as an example to illustrate the important role of DNA repair genes/pathways in the development and treatment of this malignancy, and demonstrate the application of a functional DNA repair assay, host cell reactivation (HCR), in cancer research.

2.3 DNA repair activity is a critical determinant for efficacy of anticancer treatment using chemotherapy or radiotherapy

The cell-killing mechanisms of radiotherapy and most regimens of chemotherapy are dependent on the induction of severe DNA damages, which result in apoptosis of cancer cells. Therefore, the DNA repair activity of cancer cells can play an important role in modulating patient's response to these anticancer treatments. For example, the platinum-based anticancer chemical, cisplatin is one of the most popular DNA-damaging chemotherapeutic drugs used in clinical management. It causes DNA adducts by interstrand crosslinking, which is repaired by a combination of NER and HR (Helleday et al., 2008; Miyagawa, 2008). Mutations of NER genes, such as *XPF* or *ERCC1*, may increase the sensitivity of cells toward cisplatin (Martin et al., 2008; Saldivar et al., 2007). In contrast, elevated expression of NER genes usually confers resistance to chemotherapy using DNA-damaging regimens. The expression level of *BRCA1*, which plays a primary role in HR and may has a regulatory role in NER (Hartman & Ford, 2002; Takimoto et al., 2002), is also correlated with chemotherapy efficacy. It has been shown that cells with reduced or inactivated *BRCA1* are more sensitive to cisplatin but, in contrast, are resistant to taxanes, the microtubule-interfering drugs (Husain et al., 1998; Lafarge et al., 2001; Mullan et al., 2001). Overexpression of *RAD51*, a member of BRCA/FA complex involved in HR, is also correlated with cisplatin resistance (Bhattacharyya et al., 2000). For *ATM*, an *in vitro* study showed that partial loss of distal 11q (*ATM* locus) was associated with decreased IR sensitivity in head and neck cancer cell lines (Parikh et al., 2007). Therefore, understanding the status of DNA repair genes/activity is thought to be important for the selection of appropriate chemotherapeutic regimens and may have a great impact on the clinical treatment as well as the patient's outcome.

3. Head and neck cancer

Head and neck squamous cell carcinoma (HNSCC) is the most popular head and neck cancer and is the sixth most common cancer in the world. They include malignancies originated from the epithelia of larynx, pharynx, oral and nasal cavities.

3.1 Some HNSCC risk factors are able to inhibit DNA repair

Epidemiological evidences have demonstrated that alcohol drinking, betel quid (BQ) chewing (especially in South Asia and South-West Pacific area including Taiwan), cigarette smoking, and infection of human papillomavirus are risk factors for HNSCC development (Haddad & Shin, 2008; IARC, 2004). The carcinogenicity of betel nut has been approved by the International Agency for Research on Cancer (IARC), a WHO organization, in 2004 (IARC, 2004), although the molecular mechanism underlying its carcinogenicity is not fully elucidated. In this regard, we have explored the possible effect of arecoline, a major alkaloid in betel nut, on DNA repair activity using HCR. We found that arecoline could inhibit the repair of UV-induced DNA damages, at least partly, through inactivating p53's expression and transactivation activity (Tsai et al., 2008). Besides, we also showed that arecoline could affect mitotic spindles and deregulated mitotic checkpoint, another key guardian of genome integrity (Wang et al., 2010). These results provide molecular explanation for BQ-associated carcinogenicity that has been shown previously by an increase of mitosis errors and micronucleus (MN) in mammalian cells (Lin, 2010). Micronucleus is a typical sign of GIN and is derived from either DNA strand breaks (clastogenic effect) or whole chromosome lagging during mitosis (aneugenic effect) (Norppa & Falck, 2003).

Epidemiological studies also show that the probability of HNSCC development is synergistically increased by simultaneous exposure of BQ, cigarette, and alcohols (Ko et al., 1995; Lee et al., 2005). Regarding the carcinogenic role of cigarette on the aspect of DNA repair, we also found that benzo(a)pyrene (BaP), an important carcinogen in cigarette (IARC, 2010), exhibited negative effects on DNA repair (Lin et al., 2011 manuscript in preparation). The mechanistic study regarding the synergistic effect of arecoline and BaP on regulating DNA repair, especially via p53- and aryl hydrocarbon receptor-dependent pathway, is worthy to be investigated further.

3.2 Alterations of DNA repair genes/activity in HNSCC and the relationship with HNSCC development, treatment, as well as patient's outcome

GIN is a hallmark of most human malignancies including HNSCC that elevated microsatellite instability, aneuploidy and various genomic alterations have been found by genome-wide analyses (Bockmuhl et al., 1996; Brieger et al., 2003; Friedlander, 2001; Partridge et al., 1999; Sparano et al., 2006), suggesting that GIN may be involved in the development of HNSCC. Some studies also show that DNA repair activity is reduced in the peripheral blood cells of HNSCC patients when compared with normal individuals (Cheng et al., 1998; Paz-Elizur et al., 2006), implying that altered DNA repair genes and/or activity may play a critical role in the development of HNSCC.

Studies using comparative genomic hybridization (CGH) have shown that gene copy numbers at chromosome 11q22-23 (*ATM* locus) are frequently lost in HNSCC (Bockmuhl et al., 1996; Brieger et al., 2003; van den Broek et al., 2007). Lazar et al. also showed loss of heterozygosity (LOH) at 11q23 in 25% (13/52) of primary HNSCC (Lazar et al., 1998). In addition, we have reported that *ATM* mRNA is down-regulated in 81.3% (65/80) of laryngeal and pharyngeal cancers, and further show that lower *ATM* expression (tumor/normal < 0.3) was an independent risk factor for patient's survival (Lee et al., 2011). This is the first study showing that *ATM* expression is a valuable prognostic marker for HNSCC. One study also shows an absent or reduced ATM protein expression in 31.25% (10/32) of oral cancer (He et al., 2008). These results suggest that alteration of *ATM*, either in gene sequence or in expression level may be associated with HNSCC.

Previous investigations showed that LOH of chromosome 17q (*BRCA1* locus) were found in 35% to 56% of laryngeal cancer (Kiaris et al., 1995; Rizos et al., 1998). In contrast, studies using CGH found an overrepresentation of 17q in 9% to 47% of HNSCC (Bockmuhl et al., 1996; Brieger et al., 2003), and one array-CGH reported the gain of 17q21 in 33% (7/21) of oral cancer (Sparano et al., 2006). These controversial results by genome-wide analyses may be due to the physically close localization of *ERBB2* (HER-2/neu) oncogene and the results need to be clarified by specifically looking at the *BRCA1* gene locus. Regarding the expression of *BRCA1* in HNSCC, one study showed that BRCA1 immunostaining positivity was lost in 34% (26/77) of tongue cancers, which might be correlated with early-stage tumor progression (Vora et al., 2003).

The results of genome-wide studies also suggest that genetic alterations at *RAD51* (15q15.1) and *XPC* (3p25) loci may be present in HNSCC (Bockmuhl et al., 1996; Brieger et al., 2003; Partridge et al., 1999; Sparano et al., 2006; van den Broek et al., 2007). Altered RAD51 protein expression has been reported by one pilot study with twelve head and neck cancer patients (Connell et al., 2006). The patients with high RAD51 protein levels in their pre-treatment tumor biopsies demonstrate poorer cancer-specific survival rates than those with lower RAD51 levels (33.3% vs. 88.9% at 2 years; $P = 0.025$). These results suggest that RAD51 expression may influence the outcome of with head and neck cancer patients who receive chemotherapy and radiotherapy (Connell et al., 2006). Other reports regarding altered expression of DNA repair genes in HNSCC include Ku80 (Chang et al., 2006), NBN and ERCC1 (Hsu et al., 2010; Yang et al., 2006). It has been shown that ERCC1 expression is associated with cisplatin resistance (Handra-Luca et al., 2007; Hsu et al., 2010) and NBN is correlated with outcome of advanced HNSCC patients (Yang et al., 2006). Inactivation of the BRCA/FA pathway via promoter methylation has also been described in HNSCC, and may be related to tobacco and alcohol exposure and survival of these patients (Marsit et al., 2004).

3.3 Treatment of HNSCC

Since HNSCC and its treatment can affect important physiological functions, such as speaking, breathing, and swallowing, it is important for choosing the appropriate treatment that not only cures but also benefits to the preservation of organs, physiological functions, and quality of life. The standard treatment for resectable HNSCC is surgical resection with or without postoperative concurrent chemotherapy (cisplatin plus 5-fluorouracil) and radiotherapy (CCRT). Around two-thirds of HNSCC are in advanced stage at time of diagnosis (Specenier & Vermorken, 2009). The majority of these patients with advanced stage tumors finally relapse locoregionally or at distant sites. These patients are usually qualified for palliative treatment only. Recent advances in using cetuximab (anti-EGFR) to prolong patient's survival time in locally advanced HNSCC is a big, but still not a fully satisfied progress (Vermorken et al., 2008). The use of docetaxel (a spindle poison and mitotic catastrophe inducer) can enhance the efficacy of chemotherapy using cisplatin/fluorouracil and improve slightly the overall survival rates of HNSCC patients (Hitt et al., 2005; Posner et al., 2007; Vermorken et al., 2007). These results suggest that a combination regimen exploiting different cell-killing mechanisms may be superior to monotherapy. However, an ideal combination regimen with lower adverse and side effects for efficient treatment of HNSCC is still under looking for.

3.4 Understanding the status of DNA repair genes in HNSCC is important for design of an effective therapeutic strategy for this malignancy

Since DNA repair genes/activity play a key role in cancer development and treatment, understanding their expression and genomic/functional alterations may facilitate the

identification of new predictive or prognostic markers and new therapeutic targets for treatment of HNSCC. For example, recent studies using the strategy of synthetic lethal interaction (SLI) to improve efficacy of cancer treatment have become an attractive strategy (Helleday et al., 2008). Cancer cells that can survive from innumerable genetic alterations are largely dependent on the activities of multiple DNA repair pathways. However, cancer cells may also be defective in certain DNA repair pathway that is inherent or arises during tumorigenesis. Therefore, inhibition of one DNA repair pathway may increase selectively killing of cancer cells that already have another defective DNA repair pathway. For examples, some clinical trials have shown the efficient killing of *BRCA1*- or *BRCA2*-defective cancer cells (with defective HR repair) by using *PARP1* inhibitors, which block BER pathway (Annunziata & O'Shaughnessy, 2010; Bryant et al., 2005; Farmer et al., 2005; Underhill et al., 2010). Notably, such kind of treatment is less toxic than conventional radiotherapy and chemotherapy. This may benefit to organ preservation of HNSCC patients if one can identify SLI targets (DNA repair genes are good candidates) and develop corresponding regimens for treatment. For this reason, some clinical trials are ongoing to examine the efficacy of anticancer treatments by modulating DNA repair activities that are involved in different DNA repair pathways (Bolderson et al., 2009; Helleday, 2010; Helleday et al., 2008).

4. Host cell reactivation (HCR) assay

As mentioned above, DNA repair activity plays a critical role in maintaining genome integrity. Regardless the alterations of DNA repair genes at the levels of gene expression or DNA sequence, measurement of DNA repair activity can reflect the overall biological effects that are as consequences of these molecular changes and/or anticancer drug responses. Here we describe an easy and fast functional assay (HCR) to evaluate cellular DNA repair activity *in vivo*. This method uses a plasmid that can produce luciferase in mammalian cells as a reporter. We choose luciferase as a reporter since its characteristics of high sensitivity and wide dynamic linear range for quantification. Of course, other commonly used reporters, such as chloramphenicol acetyltransferase (CAT), secreted alkaline phosphatase (SEAP) or green fluorescent protein (GFP) can also be used.

The reporter is damaged *in vitro* first and is transfected into host cells. If the damaged reporter plasmid can be repaired in the host cells, the luciferase will be re-expressed. Otherwise, the luciferase activity will be much lower than that transfected with undamaged control plasmid. By this way, one can determine the DNA repair capacity by simply measuring luciferase activity. The reporter plasmid can be damaged using various methods such as UV, chemicals or restriction enzymes and serve as substrates for different DNA repair pathways. In this chapter, we will demonstrate the use of HCR in evaluating DNA repair capacities via NER, HR and NHEJ pathways.

4.1 HCR for NER

NER is responsible for the repair of bulky DNA lesions induced by UV and a lot of anticancer drugs. Here we use UV as a method to damage a luciferase reporter plasmid. Other chemicals (such as cisplatin) that cause bulky DNA adducts can also be used.

4.1.1 Materials

1. The reporter plasmid: pCMV-Luc (Liu et al., 2004). The firefly luciferase is driven by the cytomegalovirus (CMV) immediate early (IE) gene promoter.

2. The internal control plasmid used for calibrating transfection efficiency: pRL-CMV (Promega, Cat. No. E2261). The *Renilla* luciferase is driven by the CMV IE promoter.
3. Transfection reagents: Lipofectamine™ 2000 (Invitrogen, Cat. No. 11668) and Opti-MEM® I reduced serum medium (Invitrogen, Cat. No. 31985).
4. The Dual-Glo™ Luciferase assay system (Promega, Cat. No. E2940) for analyzing firefly and *Renilla* luciferase activities.

4.1.2 Substrate preparation for NER

1. Amplify the pCMV-Luc and pRL-CMV plasmids in *E. coli* (Fig. 1A).
2. Harvest bacteria by centrifugation at 13,000 rpm for 15 minutes, discard the supernatant completely and purify plasmids using the Plasmid Midi Kit (Geneaid, Cat. No. PI025).
3. Determine plasmid DNA concentration and purity by measuring the absorbance at 260 nm and 280 nm with a UV spectrophotometer.
4. Prepare UV-damaged luciferase reporter plasmid (pCMV-Luc) with a UV-crosslinker (CL-1000, UVP). The plasmids are placed within inner side of an opened eppendorf lid (Fig. 1B) or 35 mm petri dish without lid. The UV dose for irradiation is dependent on cell types because of differential intrinsic DNA repair capacities of various cells. We use 1000 J/m^2 for 293 (human embryonic kidney), Beas-2B (human bronchial epithelium), H1299 (human lung cancer), HEp-2 (human laryngeal cancer), SAS, Ca9-22, (human oral cancer) and 500 J/m^2 for KB (human oral cancer) cells. It is important to keep above parameters (the same plasmid amount in a fixed volume for UV irradiation) consistently in each experiment, or prepare an enough quantity of UV-dameged plasmids that can be stored in aliquots at -80°C once for all experiments.

4.1.3 Transfection

1. The HEp-2 cells (6×10^4) are seeded in 24-well plates 24 h prior to transfection (the appropriate cell numbers for seeding are dependent on cell types).
2. Prepare DNA-Lipofectamine™ 2000 (Invitrogen) complexes for each sample as follows:
   a. Add 0.5 μg of UV-damaged or undamaged (serve as a control) pCMV-Luc together with 0.05 μg of internal control plasmid (pRL-CMV, Promega) in 50 μl of Opti-MEM (Invitrogen) medium, mix gently.
   b. Mix 1 μl Lipofectamine™ 2000 gently in 50 μl of Opti-MEM medium and incubate for 5 minutes at room temperature.
   c. Combine the diluted DNA with the diluted Lipofectamine™ 2000 (total volume is 100 μl). Mix gently and incubate for 20 min at room temperature to allow the formation of DNA-Lipofectamine™ 2000 complexes.
3. Add the 100 μl of DNA-Lipofectamine™ 2000 complexes to each well and incubate at 37°C in a CO_2 incubator for 6 h. Then the cells can be treated with toxicants (such as arecoline) or anticancer drugs for another 24 h. Note: cells can be treated with toxicants or anticancer drugs prior to transfection that is dependent on experimental design.

4.1.4 Dual-luciferase assay

1. After 24 h post-transfection, cells are harvested in 100 μl (adjustable) lysis buffer (0.1 M HEPES, pH 7.8, 1% Triton X-100, 1 mM $CaCl_2$ and 1 mM $MgCl_2$) with cell scrapers.
2. The cell lysates are transferred to eppendorf tubes and centrifuged at 13,000 rpm at 20°C for one minute.

3. The supernatants (50 μl, adjustable) are transferred into a 96-well plate and 20 μl of Dual-Glo™ Luciferase Reagent (Promega) are added to each well.
4. Ten minutes later, the firefly luminescence is measured by a microplate luminometer (Centro LB 960, Berthold, Bad Wildbad, Germany).
5. Add 20 μl of Dual-Glo™ Stop & Glo® Reagent (Promega) to each well and wait for 10 minutes, then the *Renilla* luminescence is read.
6. The transfection efficiency-adjusted firefly luciferase activity is obtained by dividing the *Renilla* luciferase activity.

4.1.5 Representation of NER activity by HCR assay

Since the pCMV-Luc is damaged by UV, the DNA repair activity (responsible to UV) can be represented as the *Renilla*–calibrated firefly luciferase activity derived from UV-damaged pCMV-Luc verse to those from undamaged pCMV-Luc. By this way, one can compare the effects of various environmental toxicants on cellular DNA repair capacity. For example, an inhibitory effect of arecoline on the repair of UV-damaged pCMV-Luc can be found by using HCR assay (Fig. 1C).

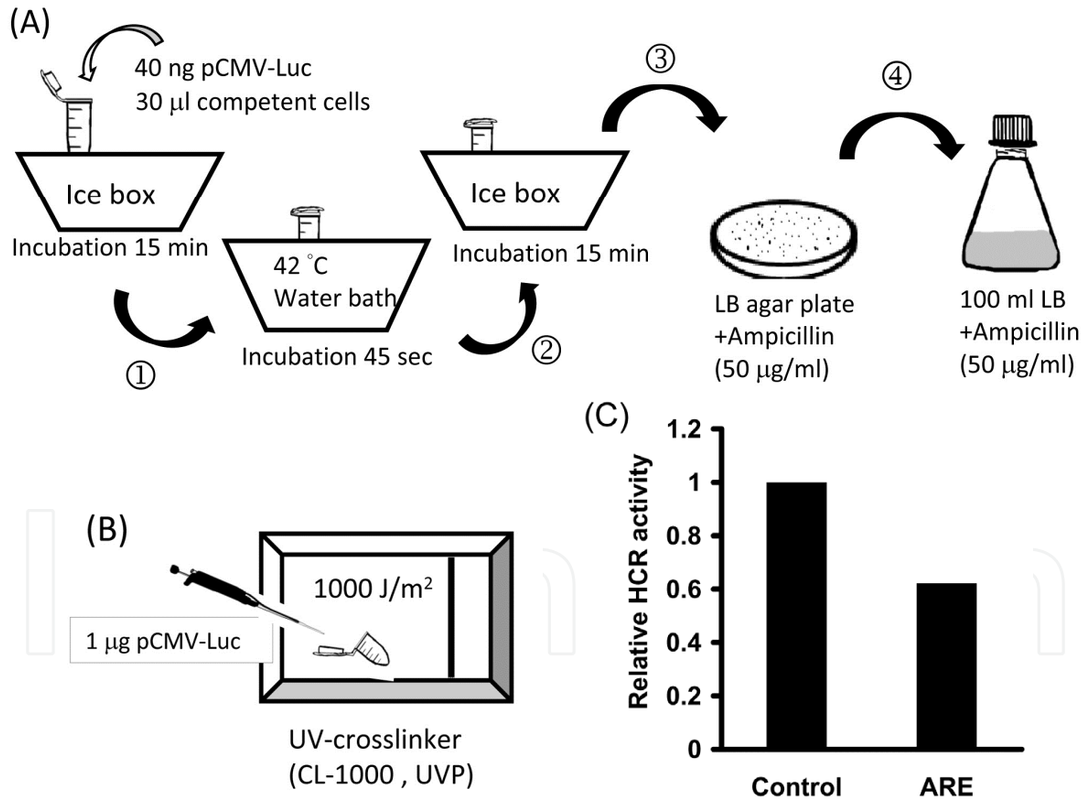

Fig. 1. Schematic illustration of host cell reactivation (HCR) assay for examination of nucleotide excision repair.
(A) The reporter plasmid pCMV-Luc is prepared in *E. coli*.
(B) The pCMV-Luc is damaged by 1000 J/m^2 of UV light.
(C) Comparison of the effects of arecoline (ARE, 0.3 mM) and its vehicle (distilled water) on the repair of UV-damaged pCMV-Luc in HEp-2 cells.

4.2 HCR for HR repair

HR is a reliable mechanism to accurately repair DNA double strand breaks. Here we use PCR to generate two overlapping DNA fragments that contain i) CMV IE promoter and 5′-part of *Renilla* luciferase gene, ii) 3′-part of *Renilla* luciferase gene and poly-A tail sequence from pRL-CMV (Fig. 2A) and serve as substrates for HR (Fig. 2B). The two overlapping DNA fragments can also be produced by restriction enzyme digestion and gel elution.

4.2.1 Materials

1. Plasmids: pRL-CMV (Promega, Cat. No. E2261) and pCMV-Luc (Liu et al., 2004).
2. PCR primers: RL_1: 5′-AGA TCT TCA ATA TTG GCC ATT AGC; RL_2: 5′-TTC TTA TTT ATG GCG ACA TGT TGT; RL_3: 5′-ACG AGG CCA TGA TAA TGT TGG ACG; RL_4: 5′-CTT ATC GAT TTT ACC ACA TTT GTA.
3. DNA Polymerase: *Ex Taq*™ Polymerase (Takara, Cat No. RR001A).
4. Gel-M™ Gel Extraction System (Viogene, Cat No. EG1002).

4.2.2 Substrate preparation for HR repair

1. Set up PCR reaction as below:

Reaction mixtures	HR_13 (μl)	HR_24 (μl)
10×PCR buffer	5	5
dNTP (2.5 mM)	4	4
RL_1 primer (10 μM)	2	-
RL_3 primer (10 μM)	2	-
RL_2 primer (10 μM)	-	2
RL_4 primer (10 μM)	-	2
ExTaq polymerase (5u/μl)	0.25	0.25
Template (40 ng/μl)	1	1
DNA/RNAase free- H_2O	35.75	35.75
Total volume (μl)	50	50

2. Incubate the PCR reaction mixtures at 94°C for 2 min, then run for 30 cycles of amplification (94°C, 45 sec; 55°C, 1 min; 72°C, 1 min) and additional extension step at 72°C for 5 min.
3. Purify the PCR products of HR13 fragments (1730 bp, containing CMV IE promoter and 5′-part of *Renilla* luciferase gene) and HR24 fregments (1023 bp, containing 3′-part of *Renilla* luciferase gene and poly-A tail) from 0.8% agarose gels (Fig. 2C) using the Gel-M™ Gel Extraction System kit (Viogene).
4. Determine DNA concentration and purity by measuring the absorbance at 260 nm and 280 nm with a UV spectrophotometer. Dilute the HR13-PCR products to 17 ng/μl and HR24-PCR products to 10 ng/μl with distilled H_2O to make the molar ratio of HR13:HR24 = 1:1, by which the same volume of the two DNA fragments can be used for transfection. Store the purified DNA in aliquots at -20°C.

Alternative: the two DNA fragments used for HR can also be generated by using a combination of restriction endonucleases *Bgl*II/*Nhe*I and *Pst*I/*Bam*HI for pRL-CMV (Progema). The use of former restriction enzymes will produce a DNA fragment containing

CMV IE promoter and 5′-part of *Renilla* luciferase gene, the later ones result in 3′-part of *Renilla* luciferase gene and the poly-A signal. These two DNA fragments contain a 222-bp overlapping region for recombination (Fig. 2D).

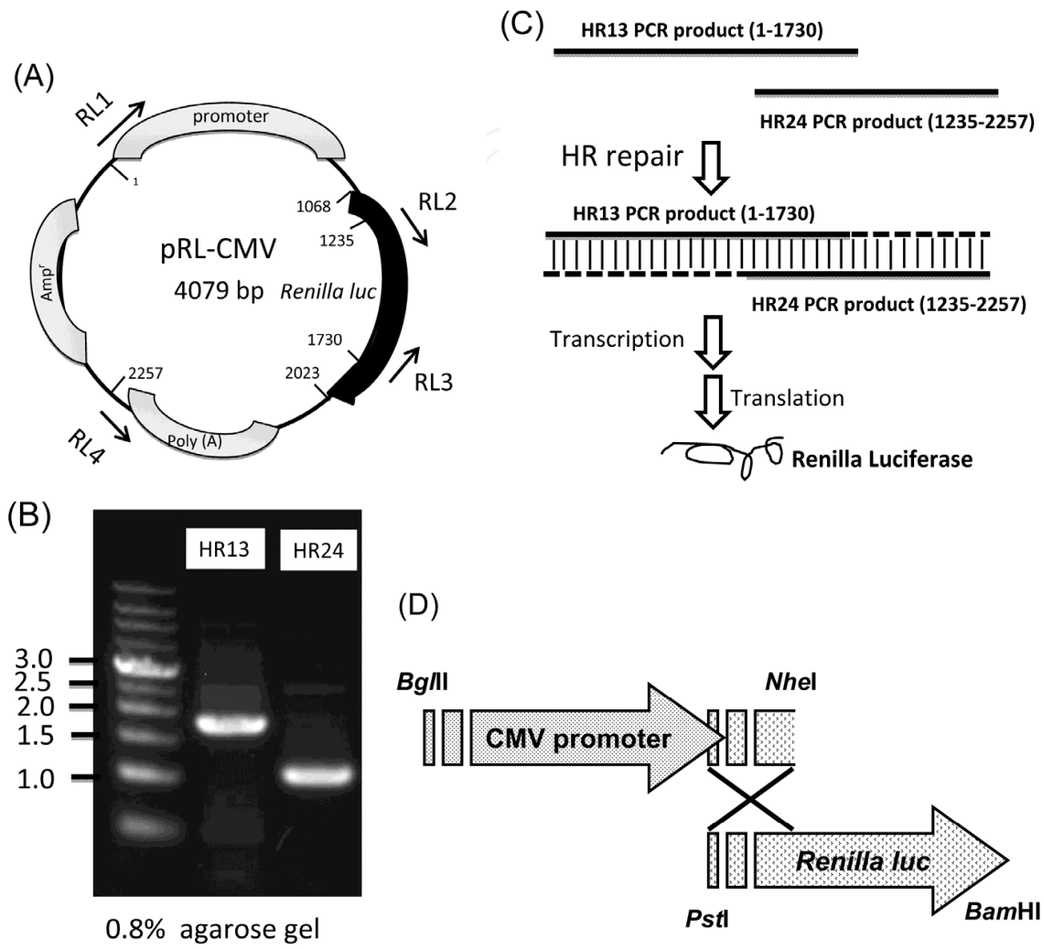

Fig. 2. Schematic representation of substrate preparation for HCR assay of homologous recombination (HR) repair.
(A) Location of PCR primers on the pRL-CMV reporter plasmid.
(B) Agarose gel electrophoresis of the PCR products, which serve as HR substrates.
(C) Homologous recombination of the two PCR fragments results in expression of luciferase.
(D) An alternative way to produce DNA fragments for HR by using restriction enzymes.

4.2.3 Transfection, dual-luciferase assay and representation of HR repair activity

1. The HEp-2 cells (6×10^4) are seeded in 24-well plates 24 h prior to transfection (the appropriate cell numbers for seeding are dependent on cell types).
2. Prepare DNA-Lipofectamine™ 2000 (Invitrogen) complexes for each sample as follows:
   a. Add 4 μl each of HR13 and HR24 DNA fragments together with 0.25 μg of internal control plasmid (pCMV-Luc) in 50 μl of Opti-MEM (Invitrogen) medium, mix gently.
   b. Mix 1 μl Lipofectamine™ 2000 gently in 50 μl of Opti-MEM medium and incubate for 5 minutes at room temperature.

c. Combine the diluted DNA with the diluted Lipofectamine™ 2000 (total volume is 100 µl). Mix gently and incubate for 20 min at room temperature to allow the formation of DNA-Lipofectamine™ 2000 complexes.

3. Add the 100 µl of DNA-Lipofectamine™ 2000 complexes to each well and incubate at 37°C in a CO_2 incubator for 6 h. Then the cells can be treated with toxicants (such as areca nut extracts, ANE) or anticancer drugs for another 24 h. Note: cells can be treated with toxicants or anticancer drugs prior to transfection that is dependent on experimental design.
4. Perform dual-luciferase assay as section 4.1.4.
5. Determine the HR activity by comparing the firefly luciferase-calibrated *Renilla* luciferase activities between the environmental toxicants- or anticancer drugs-treated cells and vehicle-treated control cells. For examples, the treatment of anticancer drug camptothecin (CPT, a topoisomerase I inhibitor) can potentiate HR repair activity but the areca nut extracts (ANE) repress HR repair in HEp-2 cells (Fig. 3).

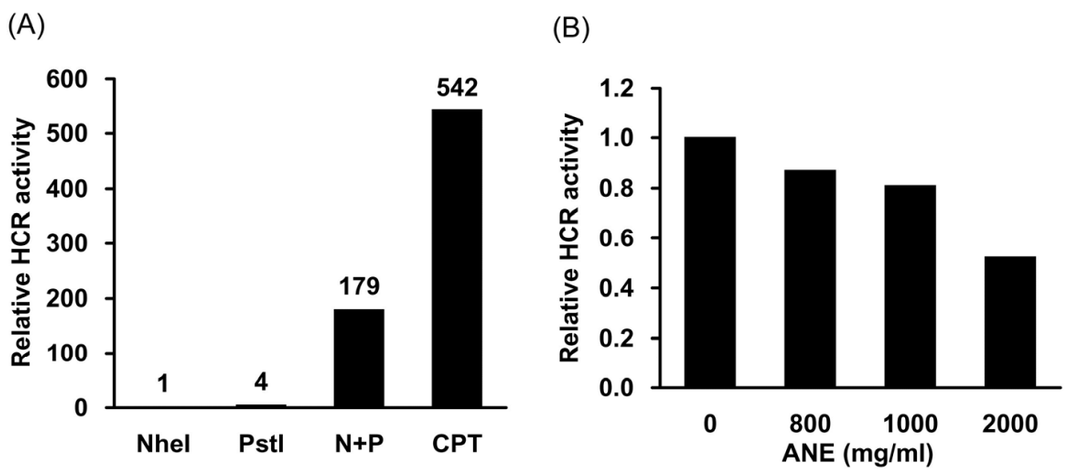

Fig. 3. The use of HCR assays in evaluating the effects of camptothecin and areca nut extracts on homologous recombination repair.
(A) Using the method illustrated in Fig. 2D, the *Renilla* luciferase activity (reflecting the HCR activity in Y-axis) can only be detected in the presence of both two DNA fragments (N+P, lane 3) but not in cells transfected with only one fragment (NheI or PstI, lanes 1 and 2). Treatment of camptothecin (CPT) stimulates HR repair efficiency in the cells (lane 4).
(B) Dose-dependent repression of HR repair activity by areca nut extracts (ANE).

4.3 HCR for NHEJ repair

NHEJ is another DNA repair mechanism responsible to DSB. Unlike HR repair using sister-chromatids as templates, NHEJ directly joins the broken DNA ends by trimming a few nucleotides on the ends. Therefore, it is thought as an error-prone repair system. In this regard, we prepare two kinds of reporter DNA substrates that are suitable for analyzing the precise and overall NHEJ repair activities, respectively.
For overall NHEJ repair, pRL-CMV is linearized with *Hin*dIII that cuts the flanking sequence between CMV promoter and the Renilla luciferase coding sequence. The luciferase will express after re-ligation regardless the loss of some nucleotides. For examining precise

NHEJ, *Afl* III that digests the coding region of *Renilla* luciferase gene is used and the luciferase can only be expressed after exact repair (Fig. 4A). The linearized reporter DNA fragments are purified, transfected into host cells and examined for luciferase activity as described above. Below is an example of evaluating the effect of areca nut extracts on precise and overall NHEJ repair (Fig. 4B).

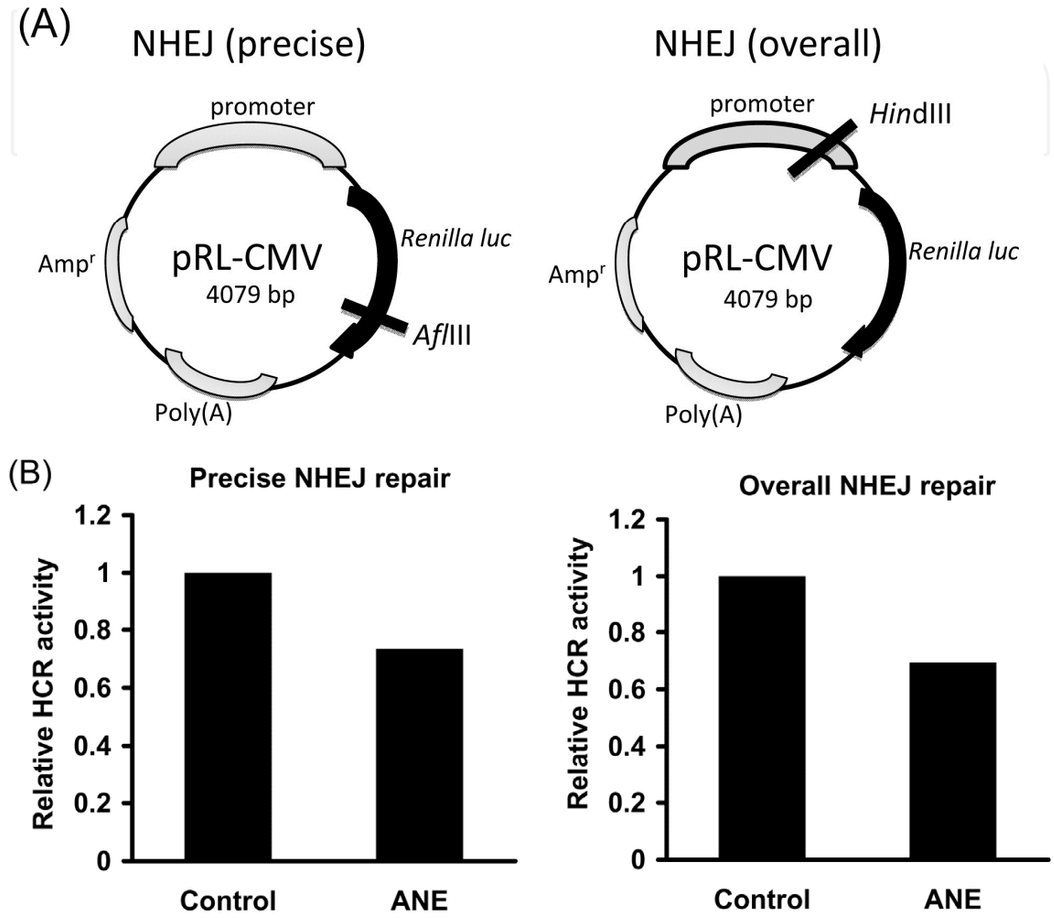

Fig. 4. HCR assay for non-homologous end-joining (NHEJ) repair.
(A) The *Afl* III-digested pRL-CMV is used as a substrate for analyzing precise NHEJ repair activity because of the need of exact joining of the *Renilla* luciferase coding sequence. For overall NHEJ, *Hin*d III that cuts the flanking sequence between CMV promoter and the *Renilla* luciferase gene is used. The expression of luciferase is not affected by loss of a few nucleotides in this region during the end-joining process.
(B) The effect of areca nut extracts (ANE, 800 mg/ml for 24 h) on precise (left panel) and overall (right panel) NHEJ repair.

5. Conclusion

DNA repair genes play a pivotal role in the maintenance of genome integrity. Alterations of various DNA repair genes, either in gene sequence/structure or in gene expression, are frequently found in most of human malignancies. Since DNA repair activity is able to

modulate cellular response to DNA-damaging anticancer drugs, alterations of DNA repair genes may be involved in the development of resistance to chemotherapy and radiotherapy. In addition, DNA repair activity plays an important role in preventing the mutagenicity and cytotoxicity induced by numerous environmental carcinogens and toxicants. Cells with reduced DNA repair activity may thus be prone to pathological transformation. Therefore, examining the DNA repair activity of a cell can help us to understand the probability of cellular tumorigenicity associated with exposure of environmental carcinogens and is able to assess the responses of various regimens of anticancer treatment. HCR assay is an easy and fast functional assay that can be applied to investigate several DNA repair pathways and is one of the most useful methods for evaluating cellular DNA repair activity *in vivo*.

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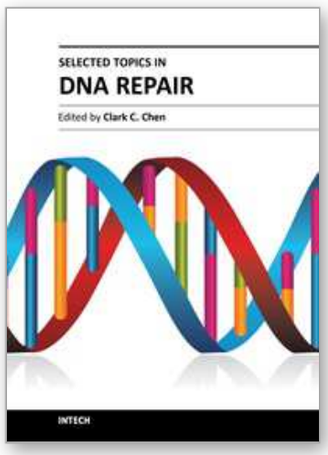

Selected Topics in DNA Repair

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This book is intended for students and scientists working in the field of DNA repair, focusing on a number of topics ranging from DNA damaging agents and mechanistic insights to methods in DNA repair and insights into therapeutic strategies. These topics demonstrate how scientific ideas are developed, tested, dialogued, and matured as it is meant to discuss key concepts in DNA repair. The book should serve as a supplementary text in courses and seminars as well as a general reference for biologists with an interest in DNA repair.

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