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Inter-individual variation in DNA repair capacity: a need for multi-pathway functional assays to promote translational DNA repair research.

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Abstract

Why does a constant barrage of DNA damage lead to disease in some individuals, while others remain healthy? This article surveys current work addressing the implications of inter-individual variation in DNA repair capacity for human health, and discusses the status of DNA repair assays as potential clinical tools for personalized prevention or treatment of disease. In particular, we highlight research showing that there are significant inter-individual variations in DNA Repair Capacity (DRC), and that measuring these differences provides important biological insight regarding disease susceptibility and cancer treatment efficacy. We emphasize work showing that it is important to measure repair capacity in multiple pathways, and that functional assays are required to fill a gap left by genome wide association studies, global gene expression and proteomics. Finally, we discuss research that will be needed to overcome barriers that currently limit the use of DNA repair assays in the clinic.

Keywords

DNA repair capacity; multiplex assays; personalized disease prevention and treatment
1. Introduction

During the time it takes to read this sentence, it can be estimated that the reader's DNA will incur on the order of 10 trillion DNA lesions. Left unrepaired DNA damage has the potential to lead to mutant cells, dead cells and ensuing disease (Figure 1). The precise number and type of DNA lesions formed varies from one individual to the next in part because of differences in exposure and lifestyle, and also because of variation in metabolism and other cellular processes. Many types of DNA damage, such as abasic sites, alkylation damage, oxidative damage, mismatches, single and double strand breaks, result from normal metabolic processes. Others are induced upon exposure to environmental agents. Among the environmentally induced lesions are bulky DNA adducts, including heterocyclic amines induced by compounds in cooked foods, cyclobutane pyrimidine dimers induced by sunlight, alkylation damage from nitroso compounds in combustion products, and oxidative damage and DNA strand breaks induced by ionizing radiation from cosmic rays and radionuclides such as Radon gas. In addition, some environmental exposures such as arsenic do not directly induce DNA damage, but are thought to increase DNA damage levels both by inducing inflammation and by disrupting DNA repair (1-3) (4, 5).

Fortunately, human cells mount a robust response to DNA damage that includes at least 7 major DNA repair pathways that specialize in the repair of subsets of DNA lesions, namely direct reversal (DR), mismatch repair (MMR), nucleotide excision repair (NER), homologous recombination (HR), base excision repair (BER), single strand break repair (SSBR), non-homologous end joining (NHEJ), and Fanconi Anemia DNA crosslink repair (FANC) (Table 1). The relationship between DNA damage and DNA repair is complex; no single pathway efficiently repairs all types of DNA lesions, some lesions are substrates for more than one pathway, and evidence for extensive interactions among proteins involved in distinct pathways continues to emerge (6-11). Mutations in DNA repair genes can have profound consequences for disease risk. The classic example is that individuals with the disease Xeroderma Pigmentosum (XP) are highly prone to skin cancer because they have mutations in genes required for nucleotide excision repair (NER), which repairs bulky lesions such as those induced by UV light. These individuals are at a 2000-fold higher risk of skin cancer in sun-exposed skin (12). A variety of other diseases including neurological, developmental and immunological disorders, as well as premature aging, are associated with aberrant DNA repair in humans (Table 1) (13). Thus, it is clear that defective DNA repair caused by mutations in repair genes represents a major disease risk factor, and genetic tests are now available for the most common disease-associated mutations in DNA repair genes (14).

Intuitively, one might expect that DRC in a given pathway should vary even among individuals who do not have rare disease-associated mutations in key DNA repair genes, perhaps due to common sequence variants and epigenetic heterogeneity across populations. DRC might thus adopt a normal distribution among individuals with disease-associated DNA repair defects (red curve in Figure 2A), as well as among apparently healthy individuals in the general population (black curve in Figure 2A) (15). One might further hypothesize, based simply on interpolation (Figure 2B), that those in the general population falling to the left of the distribution would be at higher than average risk for disease, and that
they might be candidates for personalized prevention schemes. It has been over two decades since these ideas were articulated by Hsu (16), and by Grossman and Wei (15), and although a wealth of evidence for interindividual DRC differences has since emerged from multiple laboratories using various methods (Table 2), it seems fair to say that the intervening studies have not yet resulted in personalized prevention efforts. Other possible relationships between DRC and risk of disease must also be considered (Fig 2C-2F), and these will be discussed below. Inter-individual variation in DRC might also account for differing tolerance among cancer patients for cancer therapy with DNA damaging agents. Moreover, the sometimes dramatic changes in DRC in cancer cells versus non-cancer cells might be exploited for individualized treatment.

Over the last two decades, significant efforts have focused on testing the idea that measuring DRC has the potential to inform medical and clinical practice. In this review, we discuss major lines of evidence supporting the notion that there are significant interindividual differences in DRC, and further, supporting the claim that such variations are indeed associated with disease risk. We survey the experimental approaches used to measure DRC, and discuss future work that will be needed for clinical translation of functional DRC measurements.

2. Evidence for inter-individual differences in DRC from indirect measurements

Genetics

XP was the first human cancer-susceptibility disease found to be associated with a DNA repair defect, namely NER; direct in vivo DRC measurements in cells isolated from XP patients provided the critical insight that a DNA repair defect was the cause of the disease (17). Complementation studies identified numerous genes responsible for XP, providing the foundation for predicting NER defects and associated disease indirectly from genotype analysis. Thus DNA sequencing of well-characterized mutations in XP genes can be used to predict impaired DRC and increased disease susceptibility. Subsequent research has identified numerous other disease-associated rare gene mutations that cause severe defects in the MMR, NER, HR, BER, SSBR, NHEJ, and FANC pathways (Table 1), as well as defects in DNA damage surveillance (18) and tolerance pathways (19).

Common single nucleotide polymorphisms (SNPs) that are associated with disease have been identified in genes in the DR, BER, MMR, NER, HR, and NHEJ pathways (recently reviewed in (20)). In candidate gene association studies, SNPs in DNA repair genes have been associated with increased or decreased risk of many cancers including lung, colorectal, gallbladder, oral, breast, prostate, liver, ovarian, and laryngeal cancer, as well as lymphoma and squamous cell carcinoma (20). Genome wide association studies (GWAS) have revealed many additional lower penetrance disease-associated sequence variants using unbiased computational approaches (21, 22), but surprisingly few of these turn out to be DNA repair genes. This may be explained in part by the observation that the variants identified so far explain only a small portion of disease heritability. As yet unidentified DNA repair variants may contribute to the missing heritability if they are relatively rare but confer a relatively
large risk increment. Variants in DNA repair genes that confer risk could also be missed if they represent copy number variants or they have relatively small effects; further, gene-gene interactions involving DNA repair gene variants may also be missed in GWAS studies due to low statistical power (23). Moreover, most GWAS-identified variants are not located in genic regions, but rather in intergenic regions that are presumably involved in gene regulation. Increased sample sizes, better accounting for rare variants and structural variants, and better understanding of the role of regulatory variants will likely increase the ability of DNA sequence-based assessments to identify individuals with elevated disease risk. In section 3, we will discuss in detail functional assays that may complement DNA sequence-based predictors of DRC defects.

In addition to disease prevention, genome profiling for sequence variants in DNA repair genes has the potential to enable personalized disease treatment (24); it is already clear that SNPs in DNA repair genes can play a role in assessing a prognosis for patients being treated for melanoma, pancreatic, esophageal, or non-small cell lung cancer. SNPs in the following DNA repair genes have been associated with the response of patients to cancer therapy: MGMT, XPA, XPC, XPD, XPE, XPG, ERCC1, ERCC3, XRCC1, XRCC2, and XRCC3 (25-32). Polymorphisms in some DNA repair genes, such as ERCC1 and XPD, have also been associated with increased cancer therapy toxicity (33), and MGMT polymorphisms are associated with increased risk of myelodysplastic syndromes following treatment with alkylating agents (34).

Major advantages of genomic profiling include the breadth of data that can be obtained for relatively small (and steadily decreasing) investment of resources using next generation sequencing (DNAseq), conceptual simplicity, and the universality of the approach; standardized sequencing procedures foster high inter-laboratory reproducibility (35). An important limitation of studies that aim to make predictions based on DNA sequence is that, with the possible exception of CpG methylation specific PCR (MSP), one cannot know a priori how well the gene with which the sequence variant has been associated is actually expressed; indeed differential allelic gene expression is common (36). In this regard genome sequence based assays may be regarded as the least direct means of measuring function (Figure 3).

Transcriptional profiling

Transcriptional profiling has revealed that DNA repair gene expression has important consequences for disease biology. For example, studies have identified prognostic gene expression signatures in cancer cells that correlate with breast cancer survival (37, 38), breast cancer recurrence (39), and lung cancer survival (40). Tumor gene expression profiles that include DNA repair genes and correlate with cancer therapy response have also been identified (41), and in some cases tumor expression of a single DNA repair gene correlates with treatment efficacy (42-44). Moreover, gene expression profiling has been used to identify bleomycin-induced changes in DNA repair gene expression that predict bleomycin sensitivity, and low level radiation induced changes in DNA repair gene expression in (non-cancerous) human lymphocytes that can be used as biomarkers for occupational exposure to ionizing radiation (45, 46). An additional study in human lymphocytes demonstrated an
inverse correlation between radiosensitivity measured by a G2 challenge assay and expression of the $NFKB$ gene; this study also showed an association between radiosensitivity and breast cancer risk (47).

One study with human lymphoblastoid cell lines revealed that expression of a set of just 48 genes was sufficient to predict sensitivity to MNNG (48), an alkylating agent that generates the same spectrum of DNA lesions as the chemotherapeutic drugs temozolomide, dacarbazine, procarbazine and streptozotocin (49). These findings were important in several respects; first it was possible to predict MNNG sensitivity in cells derived from apparently healthy individuals using a small set of basally expressed genes, supporting the notion that gene expression patterns could be used to help predict how tumor cells will respond to therapy. Second, there were large variations in sensitivity to two different DNA kinds of alkylating agents, and it was subsequently shown that sensitivity to one agent did not accurately predict sensitivity to a second DNA damaging agent, indicating a unique response to each agent (50). One of the predictive transcripts for MNNG sensitivity encodes the methylguanine DNA methyltransferase (MGMT) protein that repairs $O^6$-alkylguanine DNA lesions by DR. This might be expected, since $O^6$-methylguanine is one of the most toxic lesions generated by MNNG. However, MGMT expression alone was a much weaker predictor than expression of the combined set of 48 genes, indicating that sensitivity to DNA damaging agents reflects the integration of numerous biological pathways. The other 47 genes included at least one other known DNA repair gene ($MUTYH$), and it is possible that the other genes identified in this study affect DRC in ways that have yet to be established.

A major strength of gene expression profiling is the relatively new ability to complement or replace quantitative real time PCR (qPCR) and microarray gene expression assays with next generation RNA sequencing (RNAseq) to generate very accurate data assessing genome wide expression levels, along with splicing information, at steadily falling costs. However, as with genomic DNA sequencing analysis, gene expression profiling is limited because it remains a relatively indirect measure of function; the presence of a transcript does not guarantee that the translation product will be correctly folded, active, appropriately modified and localized to the correct cellular compartment.

**Mutagen sensitivity assays**

Mutagen sensitivity assays, using mutagen-induced chromosome and chromatid breaks, can also provide an indirect assessment of DRC phenotype (16, 20, 51). Epidemiological studies comparing the response of lymphocytes to mutagens including bleomycin, UV-light, and benzopyrene diol epoxide (BPDE) have revealed increased sensitivity to mutagen-induced chromosomal aberrations in lymphocytes from individuals with cancer versus lymphocytes from healthy individuals. This has been borne out in patients with cutaneous melanoma, basal cell carcinoma, squamous cell carcinoma of the head and neck, plus patients with lung, breast and bladder cancers (51). Of particular interest, a prospective study found that higher bleomycin sensitivity of lymphocytes (>0.5 chromatid breaks per cell) from patients with head and neck cancer was associated with an elevated risk (hazard ratio of 1.38) of developing second, unrelated primary tumors as well as recurrence of the original cancer (52); importantly the blood cells were drawn before development of second primary or
A second prospective study found a significant association between bleomycin sensitivity in lymphoblastoid cell lines and combined risk of prostate, lung, colorectal and ovarian cancers in the patients from whom the cells were derived (53). However, this study found no significant associations between cancer risk and several other measures of DRC, including BPDE sensitivity, endogenous DNA damage levels measured by comet assays, or host cell reactivation of UV-irradiated plasmids (comet and host cell reactivation assays are discussed in detail below). These negative results may reflect the small sample size and the focus on a limited number of DNA repair pathways.

A major advantage of mutagen sensitivity assays is that, by measuring the response of whole cells to specific mutagens of interest, they integrate biological complexity, such as SNPs, gene expression, epigenetics, protein folding and cell cycle checkpoint activation pathways that may not be accounted for by other methods of measuring DRC. On the other hand, it should be noted that a shortcoming of mutagen sensitivity assays is that they do not provide specific mechanistic information with regard to the identity of the genotoxic lesion or the pathways responsible for potentially defective repair, and they are relatively labor intensive.

3. Complications associated with indirect measurements of DRC exemplified by a simple repair pathway

The challenges associated with making accurate indirect DRC measurements can be illustrated by considering the performance of available methods for estimating MGMT activity; this protein essentially represents a one-protein DNA repair pathway. SNPs that may lead to MGMT defects have been associated with increased risk of some cancers, and better prognosis following chemotherapy with alkylating agents, as might be expected if the SNP leads to inefficient DNA repair (32). However, there are many examples of cancer cells in which MGMT is epigenetically silenced due to promoter hypermethylation (43, 54, 55); in these cases the sequence of the MGMT gene would be irrelevant to prognosis because the gene is not expressed. Thus, information about promoter methylation and/or gene expression may be needed to complement information obtained from DNA sequencing.

Epigenetic MGMT silencing due to promoter CpG hypermethylation in tumors has been detected by methylation-specific PCR methods and shown to correlate with the efficacy of cancer treatment with O\textsuperscript{6}-MeG generating chemotherapeutic agents such as temozolomide or dacarbazine (56). Nevertheless, even a combination of SNP data and promoter methylation status may fail to predict function (and therefore clinical outcome) for several reasons. MGMT methylation status is sometimes not predictive of transcript levels (57, 58), and transcript levels are not informative unless the transcripts are translated and the protein stably folded and localized to the nucleus. For example, a significant fraction of the human MGMT protein is inactive in some cells (59), possibly due to posttranslational modifications (60). Furthermore, environmental exposures can alter the activity of DNA repair proteins, including MGMT (1, 2, 61). These phenomena may not be detected by the available indirect measurements of MGMT activity.

A defect in protein localization could also confound DRC assays. Mitochondria-associated OGG1 protein and activity levels are higher in the livers of old mice and in presenescence.
human fibroblasts compared to young mice and replicating human fibroblasts, respectively. However, a significant fraction of OGG1 remains inactive and sequestered in the mitochondrial outer membrane and intermembrane space, leading to accumulation of unrepaired oxidized bases in the mitochondrial DNA (62). An age-related localization defect was also observed for the mitochondrial uracil-DNA glycosylase (UNG) (62). A potential analogous localization defect for MGMT (or any other DNA repair protein) could be difficult to predict from the DNA sequence, and would not be detected by in vitro activity assays performed on whole cell lysates. In general, relatively laborious immunohistochemistry and subcellular fractionation techniques are required to detect protein localization defects.

In this section we have principally highlighted some of the pitfalls associated with making indirect measurements of DRC in the context of the simple one-protein MGMT pathway, and one might anticipate even more complex challenges for indirect measures of DRC in pathways that involve multi-protein complexes and multiple enzymatic steps (63-72). A major strength of in vivo functional DNA repair assays is the ability to integrate the complexity described above to reflect, as closely as possible, repair of genomic DNA damage. The next section discusses recent technological advances for making direct DRC measurements.

4. Evidence of inter-individual DRC differences from direct (functional) measurements

There are numerous methods for measuring DNA repair directly, and each has its strengths and weaknesses (Table 2). Some of the earliest protocols for measuring DRC, such as unscheduled DNA synthesis, removal of radiolabeled alkylation damage from genomic DNA (73, 74), and methods using antibodies specific for DNA lesions including BPDE, 8-oxoG, O6-MeG, pyrimidine dimers and cisplatin adducts (75), hold the advantage of measuring repair of genomic DNA in intact cells. While these assays have been used to detect a 3-5 fold range in inter-individual DRC (76, 77), they are relatively labor-intensive thus inhibiting their application to large-scale studies.

Activity assays with cell lysates

Pathway-specific DNA repair activity analyses in cell lysates have yielded considerable insight into inter-individual variation in DRC. An advantage of these assays is that because they measure levels of functional protein, they integrate much of the biological complexity that might confound indirect measures of DRC (Fig. 3); indeed a low correlation between enzymatic activity and mRNA levels has been documented in some cases (78). Quantitative in vitro functional assays have been developed for various steps of BER (79-83), MMR (84), DR of alkylation damage by MGMT (85), NER (86), NHEJ (87), cross-link repair (88), and HR (89).

In vitro assays with cell-free extracts prepared from human lymphocytes have so far been used to measure inter-individual differences in MGMT activity and in the efficiency of several key steps in the BER pathway. These studies revealed an approximately 10-fold
variation in MGMT activity (90), a 10-fold variation in activity of alkyladenine DNA glycosylase (AAG, a.k.a. MPG) (49, 78, 91, 92) that initiates BER of several types of alkylation damage, and a 3-fold interindividual variation in activity of 8-oxoguanine DNA glycosylase (OGG1) (78) that initiates repair of oxidative DNA damage. Measurements of the subsequent BER steps has revealed a 1.9-fold to 2.5-fold variation in AP endonuclease activity (79, 82), 1.3-fold variation in subsequent polymerase beta dependent gap filling (82), and 3.4-fold variation in DNA nick ligation (82).

Although cell-free assays are quantitative and permit specificity with regard to the type of lesion being repaired, they have some limitations. The disruption of cells necessary for cell-free analyses can lead to dissociation of protein complexes and protein unfolding, or can mask defects in protein localization that would be detected using an in vivo assay. Similarly, the assay buffers may fail to reflect the in vivo intracellular environment. In addition, the substrates, typically naked short oligonucleotides, may not fully represent the complexity of repair in chromatinized DNA.

**Comet Assays**

Comet assays provide a powerful means of measuring endogenous DNA damage, induced DNA damage, and repair of DNA damage in genomic DNA in live cells (93). The assay is named for the comet-like appearance of DNA after single cell gel electrophoresis, before and after treatment with DNA damaging agents; measuring the disappearance of DNA damage following DNA damage induction enables estimates of DNA repair kinetics. Double strand breaks are measured at neutral pH, whereas single strand breaks are measured under alkaline conditions that dissociate DNA strands. Cells may be treated with ionizing radiation or bleomycin, which directly induce strand breaks, or agents such as UV-light, BPDE, peroxides, and alkylation agents that induce DNA lesions that can be converted into strand breaks upon processing in vivo by DNA repair machinery (94, 95). Genomic DNA base damage levels can also be measured using the alkaline comet assay following treatment of permeabilized gel-embedded cells with purified lesion-specific enzymes such as Endonuclease III (thymine glycol), FPG and OGG1 (8-oxoG), T4 endonuclease V (pyrimidine dimers), AlkA (alkylation damage such as 3-methyladenine), and UNG (uracil) that convert their respective substrates to alkalai-labile abasic sites or to single strand breaks (93).

Comet assays have been used in several studies with human lymphocytes to measure interindividual differences in DRC. One study demonstrated a 4-fold inter-individual variation in BER of 8-oxoG and a 10-fold variation in NER of UV-induced damage (96). Differences in DRC provide biological insight; reduced DRC relative to healthy individuals has been associated with cancer risk in a number of studies (Table 2). An approximate 2-fold increase in endogenous DNA damage (suggestive of reduced DRC) has been observed in lymphocytes from brain cancer patients, relative to healthy individuals (97); up to 2-fold higher levels of bleomycin induced DNA damage have been observed in lymphocytes from breast cancer patients (98); and modestly reduced repair of bleomycin-induced DNA damage has been observed in lymphocytes from both breast cancer patients and non small cell lung cancer patients relative to healthy controls (98, 99). Furthermore, reduced repair of
hydrogen peroxide-induced DNA damage was found in lymphocytes from lung cancer patients (100), and a 4-fold variation in the rate of repair for ionizing radiation induced DNA damage was found, wherein lymphocytes from head and neck cancer patients were more likely to exhibit slow repair (101). The aggregate data from these studies raise the possibility that individuals with lower DRC are more prone to cancer, and might be candidates for more aggressive cancer screening.

Finally, comet assays have also been used to show that lymphocytes from patients with extreme reactions to radiation treatment, defined as grade 4 (102), repair ionizing radiation induced DNA damage with slower kinetics than lymphocytes from normal responders (103). This work suggests that it may be possible to use DRC assays to predict radiation sensitivity, and to tailor treatment based on individual tolerance.

Until recently, the labor-intensive nature and large inter-laboratory variation in analysis of comet assay data constituted a significant barrier to the application of the comet assay in large studies. However, a chip-based comet assay with automated image analysis has opened the door to such studies (104); the comet chip assay provides a high throughput platform that significantly reduces the inherent noise in the conventional comet assay. The ability to measure repair of a wide variety of types of genomic DNA damage on a single comet chip represents a major step forward. A limitation of the assay is that while there are strategies for extending the comet assay to measure many types of DNA damage, the methodology is limited to the subset of DNA lesions that either induce strand breaks or induce damage that can be converted to strand breaks.

Host Cell Reactivation Assays

Host cell reactivation (HCR) assays offer a powerful way to measure DRC in living cells. The foundation of the assay lies in the ability of transcription blocking DNA damage to impede expression of a transiently transfected reporter gene; repair restores transcription of the reporter gene, which may encode enzymes such as chloramphenicol acetyltransferase (CAT) and luciferase, or a fluorescent protein (105). A major strength of HCR assays, stemming from the in vitro generation of damaged reporter plasmid DNA, is the ability to measure the in vivo repair of specific DNA lesions in intact cells.

The same association between reduced DRC and cancer risk found using mutagen sensitivity, comet and cell free assays, was also demonstrated in several epidemiological studies using HCR assays. For most of these studies, reporter protein activity was measured in cell lysates prepared from transiently transfected human lymphocytes. Early HCR assays using UV-irradiated CAT reporter plasmids showed approximately a 10-fold range of inter-individual differences in NER capacity, with a significantly lower average DRC in lymphocytes from basal cell carcinoma patients compared to those from controls (106). HCR assays making use of UV-irradiated CAT or luciferase reporters have revealed 10-20% reduced DRC (relative to control) in lymphocytes from patients with either melanoma or non-melanoma skin cancer (107, 108). In other studies, repair of plasmids damaged with BPDE or the nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was reduced between 10 and 60% in lymphocytes from patients with lung cancer (109-112), non-small cell lung cancer (113), breast cancer (114-116), squamous cell carcinoma of the...
head and neck (117, 118), and lung adenocarcinoma (112). Furthermore, DRC below the control median was associated with an increased risk of cancer, with odds ratios ranging from 1.5 to 5.7 (51). In further studies, lymphocytes from bladder cancer patients repaired plasmids damaged with 4-aminobiphenyl with ~10% reduced efficiency (119), and ~10% reduction in repair of plasmids alkylated with dimethyl sulfate was observed in lymphocytes from patients with lung adenocarcinoma (112). Assessment of NER capacity from apparently healthy individuals has also shown a 5.6 to 11-fold range of inter-individual variation and an inverse correlation with age and adiposity (120, 121). A plasmid end-joining assay found a statistically insignificant ~6% reduced average DRC in lymphocytes from breast cancer patients, but the lowest DRC quartile was positively associated with increased cancer risk (odds ratio 2.2) (122).

Additional HCR assays, including a few with fluorescent reporters that do not require cell lysates for analysis have been developed for measuring HR (123, 124), MMR (125), BER (126), NHEJ (127), inter-strand cross link repair (7, 128, 129) and repair of oxidative damage (130, 131). A multiplexed fluorescence-based flow cytometric HCR assay (FM-HCR) that uses different colored fluorescent reporter plasmids to measure repair of multiple doses or multiple types of DNA damage in a single assay was recently developed (132). FM-HCR is less labor intensive than HCR assays that require cell lysate preparation, and uses DNA lesion-induced transcriptional mutagenesis to measure repair of specific DNA lesions, such as O6-methylguanine and 8-oxoguanine, that are bypassed by RNA polymerase and thus refractory to conventional HCR assays. An even higher throughput HCR assay that uses deep sequencing to measure and sequence reporter transcripts (HCR-seq) was also developed (132). While the epidemiological studies have so far been dominated by NER reporters, the availability of reporters for additional pathways and high throughput HCR assays should encourage future studies to examine multiple repair pathways.

HCR assays face some potential limitations. The repair of constitutively transcribed plasmid DNA measured by HCR may not accurately reflect repair of genomic DNA. However, the assays have been validated using a variety of cell lines and in primary human blood cells with DNA repair defects caused by mutated and inactivated DNA repair genes. Importantly, numerous epidemiological studies (discussed above) have confirmed that HCR assays can reproducibly measure small DRC differences in primary human tissues that are associated with disease. In further support of the notion that plasmid DNA transactions can be reflective of genomic DNA transactions, it appears that plasmids are readily complexed into a nucleosomal structure in human cells, i.e. they become chromatinized (133-136). Moreover, plasmid DNA damage induces histone modifications that affect expression of plasmid DNA (137), indicating a functional plasmid-chromatin structure.

5. The need for assays that measure DRC in more than one pathway

The majority of epidemiological studies that apply functional DRC assays have focused on a single DNA repair pathway, namely NER. However, data continue to emerge in support of the notion that DRC for more than one pathway will be required to gain maximal biological insight. Here we consider several contexts in which multiple DNA repair pathways, or the

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multiple steps within a single pathway, interact to influence disease risk or the sensitivity of cells and animals to DNA damaging agents.

**Multiple repair defects and cancer**

Treatment with S<sub>N</sub>1 type alkylating agents such as temozolomide and decarbazine generates toxic O<sup>6</sup>-MeG lesions that are repaired by MGMT. MGMT deficient cells are thus generally very sensitive to S<sub>N</sub>1 alkylating agents. However because the toxicity of O<sup>6</sup>-MeG lesions is mediated by MMR (138, 139), MGMT deficient cells can become resistant to alkylating agents if they acquire a MMR deficiency (140). This chemoresistance or tolerance mechanism would confound efforts to predict treatment efficacy based on tumor MGMT status alone, and suggests a need to measure both pathways for improved prognosis. A second example where the status of two or more pathways determines the sensitivity of cells is seen in the context of poly(ADP-ribose) polymerase (PARP) inhibitors. PARP is involved in DNA single strand break repair, and PARP inhibition potentiates DNA damage-induced cell death (141). In the absence of DNA damaging agents, cells can generally tolerate either PARP inhibition or a defect in HR. However, because HR rescues the collapsed replication forks generated when the replication machinery encounters a single strand break, treatment of HR-deficient cells with PARP inhibitors leads to a synthetic lethality (141).

Polymorphisms in multiple DNA repair pathways or multiple steps within a pathway are associated with elevated cancer risk. A study of non-small cell lung cancer patients revealed small individual hazard ratios (up to ~1.4) for polymorphisms in genes involved in NER (XPA, XPD, XPG), BER (XRCC1), and HR (XRCC2, XRCC3), but a larger hazard ratio for patients with any combination of 4 or more polymorphisms in different genes within a pathway or in different pathways (hazard ratio 1.8) (28). For pathways that involve more than one repair protein, such as NER, there is potential for an additive or synergistic effect of combining modest functional defects in multiple steps along the pathway. A recent candidate gene association study revealed that SNPs in the NER genes XPG, XPD, XPA, and XPE are associated with worse prognosis following skin cancer diagnosis; a hazard ratio of 1.26 was calculated for individuals with a variant genotype in one of the genes, but the hazard ratio increased dramatically for individuals with variants in 2 or 3 of these genes to 3.90 and 34.3, respectively (30).

Functional DRC measurements in multiple DNA repair pathways have also revealed higher risk factors than measurements in any single pathway would indicate. A breast cancer study found that combined NER deficiency (measured by an immunohistochemical assay for BPDE repair) and NHEJ deficiency (measured by a plasmid repair assay) represented a greater cancer risk factor (odds ratio 4.92) than deficiency in either pathway alone (odds ratio 1.16) (77, 122). A second case control study of both OGG1 and AAG activities showed that reduced OGG1 activity and elevated AAG activity were associated with a higher risk of lung cancer, and most important, that a combined score for the two enzyme activities was more strongly associated with cancer risk than either OGG1 or AAG activity alone (142). Recently, this study has been extended to incorporate APE1 into an integrated DNA repair score, termed “OMA” for OGG1, MPG (a.k.a AAG) and APE1; the OMA score varies over a 20-fold range and associates even more strongly with risk of lung cancer (odds ratio 5.6...
comparing individuals with the lowest to highest tertile OMA scores) (143). These results emphasize that measuring repair capacity in more than one pathway has the potential to increase biological insight and reveal stronger correlations between DRC and disease risk. It should be noted that the lower OMA scores correspond to lower levels of OGG1 and APE1 activity, but higher AAG activity, underscoring the fact that for some pathways high levels of DRC are not always protective.

Imbalanced repair and toxic repair intermediates

Evidence for potentially harmful and tissue dependent effects from higher DRC levels has emerged from the characterization of Aag-dependent alkylation sensitivity in cells and animals. Aag deficiency has been associated with sensitivity to alkylating agents in mouse embryonic stem cells, consistent with a relationship wherein risk (defined for this specific example as the risk of cell death upon exposure to an alkylating agent) in one tissue decreases with increasing DRC (Fig. 2b). However an unexpected phenotype was observed in mouse models, wherein Aag deficiency leads to extreme alkylation resistance in certain tissues (49), indicating, that for some tissues risk increases with increasing DRC (Fig. 2c). Accordingly, overexpression of Aag leads to tissue-specific alkylation sensitivity in mice (92).

A lack of proper coordination among multiple DNA repair steps (repair imbalance) has been invoked to explain increased alkylation sensitivity in cells that overexpress Aag. Aag overexpression leads to the accumulation of DNA repair intermediates (49, 144), which include 5’-deoxyribose phosphate containing single strand breaks that can trigger hyperactivation of PARP. This enzyme modifies numerous other proteins, including several in DNA repair pathways (141, 145). PARP also facilitates both repair (SSBR and BER) as well as a cell death pathway involving NAD and ATP depletion, and an energetic crisis followed by cell death (146-148). Cells and whole animals that overexpress Aag but are genetically deficient for PARP show a complete rescue of wild type sensitivity to alkylating agents, confirming that SSB-stimulated PARP hyperactivation is responsible for hypersensitivity (92).

It should be noted that this situation contrasts with pharmacological PARP inhibition that generally leads to DNA alkylating agent sensitivity. The consequences pharmacological PARP inactivation using inhibitors may differ from the consequences of genetic depletion because inhibitors can induce formation of a stable 5’dRP:PARP:Inhibitor complex at SSBs that inhibits DNA repair and blocks replication (149-151), potentially leading to double strand breaks (152, 153). Thus PARP inhibition leads to alkylation hypersensitivity in cells that accumulate SSBs, including cells that overexpress AAG (154), and polymerase beta or ligase III deficient cells (155, 156).

SSBs also accumulate in cells from individuals with the neurodegenerative diseases spinocerebellar ataxia with axonal neuropathy-1 (SCAN1) and ataxia oculomotor apraxia-1 (AOA1) (157). For SCAN1, abortive topoisomerase-I reactions lead to SSBs covalently linked to the enzyme; SCAN1 patients are deficient for the TDP1 enzyme that hydrolyzes the 3’-phosphotyrosyl bond between stalled topoisomerase-I and a SSB to facilitate repair. A related enzyme, TDP2, hydrolyzes 5’-phosphotyrosyl bonds between topoisomerase-II and
DNA at DSBs to facilitate NHEJ-dependent repair (158), and exhibits weak 3’-tyrosyl phosphodiesterase activity (159), however TDP2 has not as yet been associated with disease. AOA1 patients are deficient for APTX, an enzyme that catalyzes reversal of premature 5’-adenylation at SSBs; although 5’adenylation is required for ligation of SSBs, if this modification occurs in the absence of a free 3’ hydroxyl, ligation cannot be completed. It is of particular interest that SCAN1 and AOA1 manifest as neurodegenerative diseases, but do not predispose to cancer. To explain this disproportionate effect on terminally differentiated neurons, it has been proposed that TDP1 and APTX may be redundant in proliferating cells because alternative end processing factors and the HR pathway can resolve SSBs during replication (157).

An additional example of a phenotype caused by the accumulation of repair intermediates that depends on multiple DNA repair proteins, comes from a recent study implicating the DSB repair protein WRN in long patch BER of adenine opposite 8-oxoguanine (A:8-oxoG) (160). Cells deficient for WRN or polymerase λ are more sensitive than wild type to oxidizing agents such as hydrogen peroxide, due to inefficient BER of oxidative damage. However, WRN deficient cells and polymerase λ deficient cells that are also deficient for MUTYH exhibit wild type sensitivity to oxidizing agents, suggesting that MUTYH leads to toxic repair intermediates. Indeed, glycosylase mediated accumulation of toxic BER intermediates has also been invoked to explain sensitivity to a variety agents including alkylating agents (49, 144), ionizing radiation (161), and 5-fluorouracil (162).

**Repair competition**

Some DNA lesions are repaired by proteins from more than one of the canonical DNA repair pathways shown in Table 1 and Table 2. Multiple pathways may either complement or interfere with one another. For example, BER of 8-oxoG opposite cytosine is initiated by one of several DNA glycosylases (OGG1, NEIL1 and NIEL2) (163). Moreover, it was recently reported that proteins involved in transcription coupled NER (e.g. XPA, CSB and RNA polymerase II), also participate in an 8-oxoG repair in actively transcribed DNA (9). Another example of distinct repair proteins competing for the same lesions arises for highly mutagenic etheno base lesions (164-166). Ethenocytosine (εC) can be bound by AAG but not excised by it, and this binding interaction prevents repair by ALKBH2 (166) and possibly TDG glycosylase. As a result, individual activity levels for AAG, TDG or ALKBH2 would not provide a complete picture for the repair of εC base lesions. Finally, although agents that form inter-strand cross-links (ICL) have been widely used as chemotherapeutics, the detailed mechanisms of ICL repair are only now beginning to be understood (167). Evidence exists for replication-dependent and replication-independent ICL repair involving proteins from several DNA repair pathways including the FA, HR, and NER pathways, as well as TLS polymerases (7, 8). Recent work shows that BER and MMR play an epistatic role in mediating cisplatin sensitivity, implicating proteins from these pathways in ICL repair as well (6, 10).

**Immune dysfunction**

Because immune function involves programmed induction of multiple types of DNA damage, DNA repair proteins from multiple pathways also play a critical role in the immune
system, and some DRC defects are associated with immunodeficiency (Table 1). Numerous DSB repair proteins are required for V(D)J recombination, which takes place in both T and B lymphocytes and is essential for the development of specialized antigenic receptors known as T-cell receptors (TCR) and B-cell receptors (BCR), composed of an immunoglobulin molecule and a CD79 moiety. The process is initiated by the Rag1 and Rag2 recombinase enzymes that induce DSBs in specific recombination signal sequences flanking V, D, and J gene units. These DSBs are then repaired by NHEJ. Consequently, many human patients with NHEJ deficiencies also have V(D)J recombination defects and suffer from a particular group of diseases known as severe combined immunodeficiencies (SCID), in particular, radiosensitive SCID. SCID is characterized by impaired T and B lymphocyte differentiation that is sometimes accompanied by deficiencies in other lineages (168). Individuals with deficiencies in NHEJ proteins (DNA-PKcs (169), Artemis (170, 171), LigIV (172) and NHEJ1/XLF/Cernunnos (173)) consistently present some degree of SCID.

Proteins from several DNA repair pathways are also involved in the terminal maturation of B lymphocytes during which two additional stages of DNA modification take place after VDJ recombination in order to increase the efficiency of the humoral response (174, 175). First, class switch recombination (CSR) exchanges the immunoglobulin (Ig) constant region to modify the Ig isotype (from IgM to IgG, IgA, etc.). In the second step, somatic hypermutation (SHM) introduces sequence diversity into the Ig variable domain to provide the potential for increased antigen affinity. Both CSR and SHM are initiated by the action of activation-induced cytidine deaminase (AID). Within hotspots, AID deaminates cytosine to uracil, creating U:G mismatches. CSR is induced when the BER protein UNG excises closely opposed uracils that are further processed by APE1 to generate a DSB that triggers processing by the HR machinery. U:G pairs escaping UNG recognition can go on to be processed by the MMR machinery and subsequently form DSB in a yet unidentified manner (176). The distinct process of SHM occurs by replication bypass of uracil (in the absence of repair by MMR or BER) inducing C to A transversions, or by translesion polymerase bypass of AP sites and gaps (generated by UNG and MMR proteins, respectively). As might be expected from their involvement in the immune response, deficiencies in both BER and MMR have been implicated in improper B-cell maturation; one of the autosomal forms of CSR deficiency known as hyper-IgM (HIGM) syndromes has been ascribed to mutations in the UNG gene (177). Similarly, three patients with deficiencies in the MMR protein PMS2 were shown to be deficient in CSR (178). Immunodeficiency syndromes are associated with increased risk of cancer (179), it has been recently hypothesized that germline mutations in genes involved in V(D)J recombination, SHM and CSR play a role in the lymphomagenesis of diffuse large B cell lymphomas (180).

Our understanding of the role that DNA repair plays during normal lymphocyte maturation continues to evolve as additional interactions between DNA repair pathways are discovered. For example, recent work suggests that interactions between MMR proteins and the MBD4 DNA glycosylase may be important for efficient CSR (181). An intriguing possibility is that the pronounced DNA repair defects associated with some severe immune disorders may
presage discovery of milder immunodeficiency that can be attributed to modest defects in multiple DNA repair pathways.

The diversity of disease states and sensitivity phenotypes associated with inefficient DNA repair in more than one pathway, or in more than one step within a pathway, calls for more studies that explore multiple repair activities. Recently accumulating data discussed above suggest subtle defects in multiple DNA repair pathways might promote disease, raising the prospect that multiplexed DRC assays could be of value in clinical diagnosis, prevention and treatment of disease.


A long-term goal that motivates many of the epidemiological studies discussed herein is to eventually apply direct or indirect estimates of DRC to the personalized treatment or prevention of disease. However current clinical practice is limited to diagnostics. Genetic testing is available for known mutations in many of the genes associated with DRC defects and disease, including *BRCA1/2, MLH1, MSH2/6, p53*, and *MUTYH* (14); individuals with these mutations are advised to undergo more aggressive screening, and in some cases prophylactic surgery. T-cell chromosome breakage or aberrations following treatment with DNA damaging agents such as mitomycin C have been used as a diagnostic for Fanconi Anemia (182), and UV-induced unscheduled DNA synthesis and sister chromatid exchange assays have been used for the molecular diagnosis of XP and Blooms syndrome, respectively (183). However, because they are labor intensive and/or expensive, both genetic testing and cell-based assays are typically used only in cases where the disease is already suspected either because of pronounced symptoms or a family history of disease.

DRC Estimates may be close to finding application in cancer treatment. Relationships between DRC and improved tumor response to anticancer drugs have been reported for MMR proficiency in cisplatin, alkylating agents, and 5-fluorouracil cancer chemotherapy (140, 184-186). XRCC1 (BER) deficiency in tumors has been linked to cisplatin sensitivity (44, 187); MGMT deficiency has been linked to temozolomide and BCNU sensitivity (188, 189), and HR deficiency to PARP inhibitor sensitivity (141). A limited study suggests that functional assays could be useful for determining the maximum radiation dose that will be tolerated by a patient (103). Thus, if individuals could be pre-identified as therapy resistant, it may be possible to raise the treatment dose to improve efficacy. Despite these examples of potential clinical applications, enthusiasm for the use of DRC measurements to guide treatment decisions are dampened by concerns about assay standardization, assay reproducibility, and the lack of prospective, randomized studies. As a result, although DRC assays are used to retrospectively classify patients into good versus poor responders to cancer therapy (28, 43, 53, 187, 190, 191), these assays are not used currently to influence cancer patient management (20, 58, 192-197).
7. What is needed going forward

To speed the translation of functional DRC assays from a laboratory tool to biomedical applications, advances are needed in several areas. (i) Excepting a small number of prospective studies (52, 53, 198), virtually all studies associating DRC defects with cancer susceptibility have been retrospective, raising concerns that the observed DRC differences may reflect changes subsequent to cancer diagnosis, or to cancer treatment. Similar levels of DNA damage have been observed in lymphoblastoid cell lines derived before versus after cancer diagnosis (198), suggesting that cancer development may not alter DRC. Moreover, one line of evidence suggests that lymphocyte DRC may be altered in cancer patients due to a systemic inflammatory response to the disease (199-203), and it is also possible that cancer treatment affects DRC. There is thus a need for additional large prospective studies to confirm that the DRC was a cause, rather than an effect, of the disease, and that subsequent treatment did not cause long-term DRC changes. We advocate that as new studies are initiated, cells from patients and their tumors should be cryopreserved such that live cells can be recovered for the purpose of functional DRC assays. Currently such samples are most often preserved for DNA, RNA and protein analyses, under conditions incompatible with in vivo functional assays. (ii) To carry out large studies, high throughput quantitative assays measuring DRC in multiple pathways are needed to maximize biological insight and prognostic potential. Standardized quantitative assays will help overcome concerns about reproducibility, an issue that is especially acute for assessments that are more subjective and less quantitative DRC indicators, such as microsatellite instability (MSI), where labs use different thresholds to distinguish MSI from microsatellite stability (204). (iii) The complexity of the relationships between DRC, other pathways, genetics, epigenetics and environmental exposure suggest that complementary approaches combining several of the techniques described above and in Fig. 3 may be needed to provide a comprehensive assessment of DRC. Collaborative projects using multiple approaches would be helpful for determining which approach or combination of approaches yields the most robust DRC-based disease prediction and diagnostic potential. (iv) The suitability of lymphocytes as a surrogate tissue for DRC in other tissues requires additional testing. The numerous epidemiological studies referenced above support the utility of measuring DRC in lymphocytes to predict disease susceptibility, and some investigators have found strong correlations between DRC in lymphocytes and other tissues (205), while others have not (90). (v) DRC variation among tissues is underexplored. In both humans and mice, large tissue-specific DRC variation has been measured for some pathways, indicating that it may be necessary to measure DRC directly in the tissue of interest (92, 206, 207). For example, DRC in liver cells might be most useful in assessing liver cancer risk; however the need for an invasive biopsy represents a major barrier to tissue-specific DRC screening. A particularly promising solution to this problem would be to generate cells representative of various human tissues by differentiating induced pluripotent stem cells (iPS cells) generated from skin fibroblasts that can be obtained from a single, relatively less invasive biopsy. The commercial availability of multiple cell types from a single individual (including iPS cells) together with advancing methods of generating iPS cells from primary tissues (208), make an initial test this approach experimentally feasible. A long-term goal would be to develop methods of measuring tissue-specific DRC variation in a variety of human cell types derived
from a skin biopsy from each individual. (vi) Finally, mitochondrial DNA repair represents a relatively understudied area, and given the severe health consequences of mitochondrial DNA depletion (209), as well as accumulating evidence of relationships among mitochondrial DNA damage, mitochondrial dysfunction and disease (210-215), it appears likely that defects in mitochondrial DRC can also be linked to disease susceptibility.

8. Conclusions

The aggregate data from several decades of molecular epidemiology indicate that DRC varies significantly among individuals, and that these variations associate with disease risk. No single DNA repair pathway is universally representative of DRC in general, and the effects of variation in repair efficiency at distinct steps or in separate pathways can combine to produce surprising and sometimes counterintuitive phenotypes. There are many ways to measure DRC. However each has its strengths and weaknesses. So far a lack of standardization and clinical validation, together with the relatively low throughput and labor-intensive nature of most methods of measuring DRC have precluded the application of functional DRC assays for personalized disease prevention and treatment. However a recent burst of technical advances, including the highly automated comet chip (104), a proof of concept for integrating DRC in multiple pathways to calculate disease risk (143), and highly multiplexed HCR assays (132), support the notion that measuring DRC could become common clinical practice. To promote this transition, these emerging technologies should be further developed, standardized and validated across multiple laboratories in large (ideally prospective) epidemiological studies employing measurements of multiple DNA repair pathways.

References


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Figure 1.
DNA damage, DNA repair, and disease. The canonical role of DNA repair is to protect cells from death, mutation, and the inception of disease. As discussed in the main text, increasing DNA repair can also have the opposite effect, inducing cell death because of the potential accumulation of toxic repair intermediates. The environment and the physiology of the individual enter this diagram at two points; both factors may may increase DNA damage, or they may affect DRC either positively or negatively.
Figure 2.
Potential consequences of DRC variability. a) Both intuition and experimental data point to a distribution in DRC, shown here for a single pathway among healthy individuals (black curve), and a multimodal distribution when disease states (red curve) are included; this panel is inspired by a similar figure originally published by Grossman and Wei (15). b) The simplest assumption is that a generic representation of risk (cell death, disease diagnosis or mortality) will decrease as DRC increases. c) In some cases, such as when a level of glycosylase initiates BER leading to an accumulation of intermediates that are more toxic than the initial DNA damage, elevated DRC may be deleterious. d and e) In principle the combined influence of factors driving the relationships in panels b,c could lead to more complicated relationships between risk and DRC. f) The relationship between DRC and risk may be represented as a complex landscape that depends on DRC in more than one pathway. As discussed in section 5, multiple DRC defects can act synergistically, but can also produce surprising and counterintuitive phenotypes.
Figure 3.
Methods of assessing DRC and their limitations. The biological trajectory that runs from genes to function includes numerous intermediate steps at which a given assay may fail to predict the functional endpoint. The point along this trajectory at which a particular method may fail is indicated by color-coded bars running from left to right. Although any of the assays may accurately predict function, only functional (Comet and HCR) assays integrate the complexity of this entire trajectory into their readout.
## Table 1

<table>
<thead>
<tr>
<th>Repair pathway</th>
<th>Primary Lesions</th>
<th>Genes Associated with Disease</th>
<th>Diseases Associated</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>DR</td>
<td>O⁶-meG</td>
<td>MGMT</td>
<td>- Esophageal, Lung Cancer (216)</td>
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<td>MMR</td>
<td>Mismatches, loops</td>
<td>MSH2, MLH1, MSH6, PMS2</td>
<td>- Hereditary Non-Polyposis Colon Cancer (HNPCC) (217) (218), - Class Switch Recombination (CSR) (219) (220), - T-cell non-Hodgkin's lymphoma (T-NHL) (221)(223), - Diffuse large B-cell lymphoma (DLBCL)</td>
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<td>NER</td>
<td>Bulky adducts</td>
<td>XPA, XPB (ERCC3), XPC, XPD (ERCC2), XPE (DDB1 &amp; DDB2), XPF (ERCC4), XPG (ERCC5), ERCC1, CSA, CSB, TTDA</td>
<td>- Xeroderma Pigmentosum (XP) (12) (224), - Cockayne Syndrome (CS) (221)(223), - Trichothiodystrophy (TTD) (222)(223)</td>
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<td>HR</td>
<td>DSB</td>
<td>BRCA1, BRCA2, NBS1</td>
<td>- Breast, Prostate Cancer (225) (226), - Nijmegen Breakage Syndrome (NBS) (225)(226)</td>
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<td>BER &amp; SSBR</td>
<td>Damaged bases, SSB</td>
<td>MUTYH, UNG, OGG1, AAG, APE1, TDP1, APTX</td>
<td>- MUTYH-Associated, Polyposis (MAP) (227)(177), - Hyper-IgM syndrome (HIGM) type V (228)(157) (143), - Lung Cancer (228)(157), - Spinocerebellar ataxia with axonal neuropathy 1 (SCAN1) (228)(157), - Ataxia-oculomotor apraxia 1 (AOA1) (228)(157)</td>
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<td>NHEJ</td>
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<td>DNA-PKcs, Artemis, LigIV, NHEJ1/XLF/Cernunnos</td>
<td>- Severe Combined Immunodeficiency (SCID) (170)(172) (171)(173) (169)</td>
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<td>- Fanconi Anemia (FA) (182) (226) (8), - Nijmegen Breakage Syndrome (NBS) (169)</td>
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*DNA Repair (Amst).* Author manuscript; available in PMC 2015 July 01.
Studies in which DNA repair assays have been used to evaluate human inter-individual and/or tumor-specific variability in DRC. Publications are categorized by DNA repair pathway studied and the type of assay used. **Black** corresponds to studies in which a single repair pathway was assayed. **Blue** corresponds to studies in which two or more pathways were assayed as a consequence of a lesion being repaired by more than one pathway or a protein being involved in more than one pathway. **Red** corresponds to studies in which two or more pathways were assayed simultaneously either through sequencing of two or more genes or by the use of separate repair measurements.

<table>
<thead>
<tr>
<th>Repair pathway</th>
<th>Indirect measurements</th>
<th>Evidence of inter-individual/tumor differences in DRC</th>
<th>Direct measurements</th>
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