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Endogenously generated DNA nucleobase modifications source, and significance as possible biomarkers of malignant transformation risk, and role in anticancer therapy.

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Abstract

The DNA of all living cells undergoes continuous structural and chemical alteration, which may be derived from exogenous sources, or endogenous, metabolic pathways, such as cellular respiration, replication and DNA demethylation. It has been estimated that approximately 70,000 DNA lesions may be generated per day in a single cell, and this has been linked to a wide variety of diseases, including cancer. However, it is puzzling why potentially mutagenic DNA modifications, occurring at a similar level in different organs/tissue, may lead to organ/tissue specific cancers, or indeed non-malignant disease – what is the basis for this differential response? We suggest that it is perhaps the precise location of damage, within the genome, that is a key factor. Finally, we draw attention to the requirement for reliable methods for identification and quantification of DNA adducts/modifications, and stress the need for these assays to be fully validated. Once these prerequisites are satisfied, DNA modification measurements, may be helpful as a clinical parameter for treatment monitoring, risk group identification and development of prevention strategies.

Keywords: DNA damage, DNA repair, biomarkers, methylation, oxidative stress, 8-oxo-7,8-dihydroguanine; 5-hydroxymethylcytosine; 5-formylcytosine; 5-carboxycytosine; 5-hydroxymethyluracil; cancer;

1. Introduction

The DNA of all living cells undergoes continuous structural and chemical alteration which, in part, occurs as a consequence of fundamental metabolic pathways, such as cellular respiration, replication and DNA demethylation. Generally, these alterations result in subtle modifications of the DNA structure, such as the addition of an oxygen, or methyl group. However, the modified nucleobase may have profound functional consequences for the cell.

Cellular respiration is a source of reactive oxygen species (ROS), as is a wide variety of environmental insults, such as ionizing or ultraviolet radiation, and exposure to certain chemicals. These ROS lead to the continual modification of cellular DNA, both nuclear and mitochondrial. 8-Oxo-7,8-dihydroguanine (8-oxoGua; this is increasingly the preferred nomenclature, see Cooke et al. for the rationale [1], Figure 1), is one of a number of oxidatively modified DNA nucleobases and its measurement is widely used as a biomarker of oxidative stress. It is also the most studied ROS-derived, DNA damage product, and is often used as a primary example to illustrate how oxidatively generated lesions may exert their pathological effects.

During replication Ura, a canonical base of RNA, may be inserted into DNA, instead of Thy, resulting in Ura:Ade mispairing. It may also arise as a consequence of spontaneous deamination of Cyt (leading to Ura:Gua mispairing). Ura:Gua mis-pairs are mutagenic and may lead to C→T transitions. In contrast Ura:Ade base-pairs are not directly mutagenic, however, they may be cytotoxic, and their presence may lead to mutations when Ura is excised by uracil-DNA glycosylases (UNGs) with the resultant formation of an abasic site.

DNA methylation is involved in a range of diverse biological processes, including gene expression which, in turn, has a profound impact on cellular identity and organismal fate [2].

Abbreviations: reactive oxygen species, ROS; 8-oxo-7,8-dihydroguanine, 8-oxoGua; uracil-DNA glycosylase, UNG; ten-eleven translocation protein, TET; 5-methylcytosine, 5-mCyt; 5-hydroxymethylcytosine, 5-hmCyt; 5-formylcytosine, 5-fCyt; 5-carboxycytosine, 5-caCyt; base excision repair, BER; thymine DNA glycosylase, TDG; 5-hydroxymethylUra, 5-hmUra; activation-induced cytosine deaminase (AID); mismatch repair, MMR; deoxyuridine-triphosphatase; dUTPase; somatic hypermutation, SHM; mismatch repair, MMR; European Standards Committee on Oxidative DNA Damage, ESCODD; deoxuynucleosides, dN; 8-oxoguanine DNA glycosylase 1, OGG1; 8-oxo-7,8-dihydro-2'deoxyguanosine, 8-oxodG; lysine-specific demethylase 1, LSD1; single-strand-specific monofunctional uracil DNA glycosylase, SMUG; acute myeloid leukemia, AML; hematopoietic stem cells, HSC; two-dimensional ultra-performance liquid chromatography with tandem mass spectrometry, 2D-UPLC-MS/MS.

Recently, it was reported that Cyt methylation in cellular DNA is much more dynamic than previously thought. It was found that a family of ten-eleven translocation proteins (TET 1, 2 & 3 enzymes) are responsible for the process of active DNA demethylation (Figure 2). TETs can catalyze oxidation of 5-methylCyt (5-mCyt) to form 5-hydroxymethylCyt (5-hmCyt) and the oxidation reaction can proceed further to generate 5-formylCyt (5-fCyt) and 5-carboxyCyt (5-caCyt). 5-fCyt and 5-caCyt may be subsequently recognized and removed by base excision repair (BER), via thymine DNA glycosylase (TDG), to complete the process of active DNA demethylation (reviewed in Bhutani et al. [2] and Cadet & Wagner [3]). Recently, 5-hydroxymethylUra (5-hmUra) was shown to be generated also from thymine *via* a TET catalysed reaction [4]. It has been proposed that 5-hmCyt, and perhaps also other modifications, may serve as biomarkers of cancer risk [5].

The aim of this review is to describe the endogenous processes which surround the generation, and removal, of the most common types of DNA nucleobase modifications, namely 8-oxoGua, Ura and certain epigenetic modifications, together with the role of anticancer therapy in the generation of these modified DNA nucleobases.

2. Uracil

2.1. Sources of Ura in DNA

2.1.1. Spontaneous Cyt deamination

Under physiological conditions, spontaneous, hydrolytic deamination of Cyt occurs readily, giving rise to Ura. Lindahl estimated that between 100 and 500 Ura residues may occur per day in the DNA of a single cell as a result of this process, and that such deamination occurs primarily in single-stranded (ss) DNA regions *e.g.* transcription bubbles or replication forks [about 100-fold more rapidly than in double-stranded (ds) DNA] [6]. This finding may explain the higher rate of spontaneous Cyt deamination in *Saccharomyces cerevisiae* compared to *Escherichia coli*, since the slower rate of transcription in Eukaryotes may keep DNA transiently in the ss form for a longer period of time [6]. These findings are in a good agreement with *in vitro* studies which demonstrate that Cyt deamination is less likely to occur in dsDNA, than ssDNA [6].

Another mechanism which may contribute to Cyt deamination in mammalian cells is the enzymatic conversion of Cyt in CpG islands to 5-mCyt, catalysed by cytosine-5-methyltransferase. This reaction starts with a formation of covalent bond with the C6 position of Cyt, leading to a transient dihydropyrimidine reaction intermediate that is subject to spontaneous deamination. The next step of this reaction is the transfer of methyl group from S-

adenosylmethionine, acting as a donor, to the Cyt moiety. This kind of enzyme-induced Cyt deamination was reported to occur in abortive catalysis by cytosine-5-methyltransferase, or at very low concentrations of S-adenosylmethionine [7].

2.1.2. dUTP misincorporation into DNA

It is an intrinsic property of replicative polymerases to misincorporate dUTP into DNA (both nuclear and mitochondrial) with similar efficiency as dTTP [8], hence the degree of misincorporation is likely to depend on their concentrations/ratio in the cellular precursor pool (Figure 3). Physiological concentrations of dUTP and dTTP are estimated to be about 0.2 and 37 μM, respectively. Thus the normal intracellular ratio is well below 1:100. However, in differentiated, non-proliferating cells *e.g*. neurons, quiescent lymphocytes and macrophages, the intracellular nucleotide pools may be imbalanced with much higher levels of dUTP (reviewed in [9]). Although dUTP is a normal intermediate in thymidylate biosynthesis, its accumulation may result in extensive misincorporation into DNA, which generates a substrate for BER via Ura DNA glycosylase a.k.a Ura N-glycosylase (UDG and UNG, respectively). Repeated cycles of misincorporation and repair increase the likelihood of abasic site formation, and the number of DNA strand breaks which, in turn, may lead to cell death. UNG exists in both mitochondrial (UNG1) and nuclear (UNG2) isoforms, which are highly specific for Ura, in ss and dsDNA, along with 5-fluorouracil, a Ura analogue used in cancer therapy [9].

Based on the relative sizes of the dUTP, and dTTP pools it is estimated that about one dUTP residue per 10^4 dTTP is misincorporated into the DNA of every human cell per day [9]. Since the dUTP/dTTP ratio is crucial for preventing Ura misincorporation into DNA, dUTPs level are strictly regulated, and the enzyme deoxyuridine-triphosphatase **(**dUTPase**)** is responsible for this task. However, it should be highlighted that the product of the reaction catalyzed by dUTPase, namely dUMP, is also a necessary precursor for dTTP synthesis. Therefore, the enzyme fulfills a dual role: catalyzing dUTP hydrolysis, and hence preventing Ura incorporation into DNA; and delivering dUMP as an essential intermediate for the synthesis of the cellular dTTP pool. Reflective of there being both mitochondrial and nuclear dNTP pools, dUTPase exists in two isoforms: mitochondrial (cytoplasmic) and nuclear, both encoded for by the *dut* gene [10]. Both isoforms have similar affinities for dUTP, and are largely identical differing only in a short region of their NH_2 termini, which contains the mitochondria-targeting motif. Expression of nuclear dUTPase is proliferation-dependent such that its activity is limited mostly in dividing cells. In contrast mitochondrial dUTPase is expressed constitutively,

independent of cell cycle status [10]. The presence of nuclear and mitochondrial isoforms of both dUTPase and UNG, suggests that excluding Ura from DNA is critical for maintaining the integrity of mitochondrial as well as nuclear DNA.

2.1.3. Activation-induced Cyt deaminase (AID) and/or APOBEC deaminases family as a source of Ura in DNA

The discovery that enzymatic deamination of Cyt to Ura in the *Ig* locus in B cell lymphocytes is necessary for antibody diversification after antigen exposure, is only a relatively recent event. Activation-induced Cyt deaminase (AID) initiates the process that consequently produces substitutions of all four deoxribonucleotides which, in turn, introduces point mutations into the variable regions of *Ig* genes at a rate almost a million times higher than the spontaneous mutation rate in somatic cells [11]. This high mutation rate is responsible for the somatic hypermutation (SHM) of the *Ig* genes, and is directly involved in class switch recombination. Generally processing of Ura, formed as a result of AID activity, recruits UNG2 and MSH2-MSH6 from the BER and mismatch repair (MMR) pathways, respectively. It has been proposed that there are two distinct phases of SHM; the first depends on the activity of AID, and the second phase depends on the mutagenicity of the error prone repair of AID-induced substitutions ([12] Figure 4). Phase one occurs when UNG2 removes Ura, formed after AID, and creates an abasic site (which has no coding potential) then, in phase two, this abasic site can be filled in with any of the four nucleobases. Phase two depends mostly on the error-prone MMR, which recognizes an Ura:Gua mispair. A section of the Ura-containing strand is removed, and monoubiquinylated PCNA attracts members of a family of low-fidelity translesional DNA polymerases which contribute to nucleotide substitutions at an unprecedented level (for a detailed review see [12]).

One of the most intriguing questions is whether this high level of mutation is selectively targeted to specific regions of the antibody genes, or whether it is possible that regions outside the *Ig* locus, or genes other than the *Ig* genes that are expressed in activated B cells, or indeed genes of other cell types, undergo such a rate of mutation. Recent studies demonstrate that AID deaminates Cyt moieties in 25% of the genes expressed in mouse germinal centers of B cells [11]. However, the mutation rate of these genes is much lower than the *Ig* loci *i.e*. for *Bcl6* and *Cd83*, mutation frequencies are 20- and 40-fold lower, respectively, and for all the other genes about 100-fold lower than the *Ig* gene [11]. It was also shown that there are two levels of genome protection: (i) selective targeting of AID to specific genes, and (ii) the “safety net” of high fidelity DNA repair mechanisms which remove Ura generated by AID, just like Ura formed by other

processes [11]. Combined, these processes maintain genomic stability and prevent the initiation of pathological processes, which have been linked to modified DNA.

2.2. Aberrant AID expression and cancer

Aberrant AID expression, which can lead to genome-wide mutations in genes other than *Ig*, and in non-lymphoid cells, contribute to the genetic changes which may result in cancer. Constitutive AID expression in transgenic mice is responsible for the development of lymphomas [13]. These animals also developed tumours in the lung, liver and stomach. Furthermore, a variety of human tumours have AID-generated mutations in key oncogenes (*i.e. MYC*, *PIM1*, *RHOH*, *PAX5*) and the tumour suppressor gene *p53* [11].

It is reported that *Helicobacter pylori* infection may cause the aberrant expression of AID, acting by directly activating proinflamatory cytokines, *via* the NF-κB pathway which, in turn, triggers AID expression [14]. This suggests that AID expression, at least in human gastric epithelial cells, is regulated through activation of the NF-κB pathway. Aberrant AID expression appears to directly lead to *p53* mutations in gastric cancer [14], although the question of "how?" remains unanswered.

Aberrant AID expression has also been described in human B-cell, non-Hodgkin's lymphoma [15], human hepatocellular carcinoma and the non-cancerous liver tissues of patients with chronic hepatitis, or liver cirrhosis [16]. More recently elevated expression of APOBEC family members, mostly APOBEC3B, was linked with Cyt deamination and Cyt to Ura transitions in a multitude of cancers [17,18]. Similarly to AID deamination of Cyt, APOBEC enzymes may be responsible for a variety of mutational events [17]. In most cases Ura is removed by the UNG/BER pathway, and is replaced faithfully. However, during replication Ura may form base pairs with Ade, which in turn results in Cyt to Thy transitions. In some instances after Ura is removed, an abasic site is the endpoint, this may cause insertion of any nucleobase opposite, since this site is non-instructional (see also section 2.1.3). The abasic site may also be processed into further, which may be a source of single- and double-strand breaks, which may lead to chromosomal aberration. Notably overexpression of ABOBEC and Ura in DNA are likely major source of mutations in multiple of human cancers [17,18].

2.3. UNG deficiency may lead to carcinogenesis

As mentioned above, recognition of Ura by UNG would normally (other than in *Ig* genes) result in error-free DNA repair, thereby protecting the genome from mutations. However, in antibody diversification, the mutation rate is almost a million times higher than the spontaneous mutation rate in somatic cells. This results in high levels of repair activity, and subsequent abasic site formation, which can lead to in the insertion of a non-instructional nucleobases, and mutation fixation, leading to SHM and class switch recombination. Thus, deficiencies in UNG could lead to immunological dysregulation or, in the case of AID mis-targeting, the development of B-cell lymphomas. Indeed, in *UNG*-deficient mice, disturbances in antibody diversification occur [19], and at three months old these mice develop lymphatic hyperplasia, and in later life have a 22-fold increase risk of developing B-cell lymphomas [19].

It is reported that the genes most frequently targeted by AID (other than the *Ig* genes) are not necessarily those with the highest mutation rate. To address this seemingly contradictory finding, an appealing hypothesis has recently been put forward, which suggests that the balance between error-prone and high fidelity DNA repair determines the participation of AID-generated Ura in carcinogenesis. It is hypothesized that the genome is efficiently protected from AID-generated mutations not only by targeting the AID, but also by the high fidelity repair of some AID-generated Ura moieties. In support of this hypothesis are findings showing that at some genes displaying a high frequency of mutations (*e.g. Ig* or *Bcl6*), most of the AID-generated Ura is repaired *via* an error prone manner (i.e. MMR), while in the genes where the frequency of mutation is lower, high fidelity repair is the predominant mechanism responsible for Ura removal [11].

2.4. The background level of Ura in DNA

There are several reports concerning the determination of Ura level in DNA which substantially differ in their estimates, ranging from 3×10^3 to 4×10^6 Ura moieties per diploid genome, *i.e.* almost three orders of magnitude separate the highest from the lowest estimates [17,20]. The very wide variation in estimates of the basal level of this form of modification may depend upon cell type used and design of the study. However, as we have seen from the work of the European Standards Committee on Oxidative DNA Damage (ESCODD), the analytical methods used for quantification may also contribute significantly.

Background levels of Ura have been determined using different techniques including GC/MS and LC/MS [20]. These techniques are combined with the use of UNG to recognise and

excise Ura moiety from DNA. This raises the important question of how efficient is this enzyme in detecting and removing damage? As noted above, UNG is one of the initiating DNA glycosylases of the BER pathway, and is primarily responsible for the recognition and removal of Ura. However, it is reported that UNG exhibits a preference for Ura in ssDNA, together with Ura:Gua base pairs over Ura:Ade (reviewed in [19]). There are also results demonstrating UNG preference for Ura within a particular sequence context [21]. Therefore the use of UNG may not release, and hence GC-MS may not detect, all the potential Ura. Enzyme efficiency will also depend upon experimental conditions raising the possibility of inter-assay variation caused by differences in enzyme efficacy.

There are some experimental data which report up to 1 x 10^4 Ura residues are generated in per human genome per day [22], with the authors concluding that this is a realistic background level of Ura in DNA. As noted earlier, spontaneous deamination of Cyt should be relatively a rare event and enzymatic deamination of Cyt, under physiological conditions, is restricted only to the *Ig* locus in B lymphocytes. However, dUTP, instead of dTTP, can be easily incorporated into DNA by polymerases if the cellular ratio of the dTTP/dUTP is too low. Since mammalian DNA polymerases do not differentiate between dTTP and dUTP, the background level of Ura in DNA may primarily depend on the activity of dUTPase, which regulates the size of the dUTP pool, and UNG. Consistent with this, the expression of the nuclear forms of *dut* and *UNG2* are cell-cycle regulated with the highest expression during S phase, with low and, in the case of dUTPase almost undetectable, levels in differentiated cells [23]. The possibility also exists that, if the levels of dUTP are high, then Ura may be reincorporated during repair synthesis, despite the efforts of UNG to exclude it from the genome. Recently, using LC-MS/MS-based methodology and after exclusion of the most likely factors responsible for potential artifactual formation of Ura in DNA (e.g. contamination of reagents with deaminases), the baseline level of Ura was estimated to be in the range of 0.056 to 4.03 dU/10^6 deoxuynucleosides (dN) in cultured cells lines [24,25], 1 to 9.6 dU/10^6 dN in human leukocytes [26], 11.41 dU/10^6 dN in human colorectal cancer tissue and 12.17 dU/10^6 dN normal colon tissue from the tumour's margin [27].

3. 8-Oxo-7,8-dihydroguanine

3.1. Source and mutagenic potential of 8-oxoGua

It has been shown that free radical attack upon DNA generates a wide range of DNA modifications, among them modified DNA nucleobases. ROS attack of DNA leads to a large number of pyrimidine- and purine-derived lesions, and some of these modified DNA nucleobases

have considerable potential for downstream effects on the integrity of the genome (reviewed in [28]).

Many oxidatively generated nucleobase lesions are mutagenic, irrespective of whether they are formed *in situ*, or arise by misincorporation from the 2'-deoxyribonucleotide pool. For the most part, 8-oxoGua formed *in situ* results in G→T substitutions, whereas 8-oxo-7,8-dihydro-2'-deoxyguanosine triphosphate (8-oxodGTP), misincorporated from the oxidised dGTP pool opposite dA, produces an A→C substitution. Generally, oxidatively generated DNA nucleobase lesions are best described as weakly mutagenic, for example, 8-oxoGua has a mutation frequency of 2.5 - 4.8% in mammalian cells, although its propensity for formation, persistence and accumulation *in vivo* could give this value greater significance. Indeed, oxidation events are reported to be largely responsible for spontaneous mutagenesis (reviewed in [29]).

It is clear that, depending upon the lesion in question, one consequence of oxidised nucleobases persisting in DNA is mutation. For this reason multiple systems exist to prevent lesion formation and, should damage occur, ensure rapid lesion removal. Furthermore, the repair enzyme systems possess considerable redundancy in their substrates. Analogous to the processes which exclude Ura from DNA, the guanine oxidation, or "GO" system, first describe in *E. coli*, represents a means to prevent the persistence of 8-oxoGua in DNA [30]. The human equivalent of the GO system, at its simplest, includes involvement from human 8-oxoGua DNA glycosylase 1 (hOGG1; BER), human mutY homologue (hMYH; MMR), and human mutT homologue (hMTH1, or NUDT1; prevention of misincorporation). Working in concert, these enzyme systems remove 8-oxoGua, or native nucleobases mis-paired opposite 8-oxoGua, from genomic (nuclear and mitochondrial) DNA; or hydrolyse 8-oxodGTP to 8-oxodGMP in the dGTP pool, to prevent misincorporation by DNA polymerases. The products of these repair activities (in addition to maintenance of genomic integrity) are 8-oxoGua, unmodified nucleobases (which can be salvaged and recycled) and, as we have proposed previously, 8-oxodG [31]. Although it appears that the potential exists for these oxidised products to be erroneously reincorporated into DNA [32], the majority ultimately appear in the urine where their measurement is used as a well established, non-invasive biomarker of oxidative stress [31].

3.2. Role of oxidatively generated DNA damage in malignant transformation

DNA oxidation has a potential role in the initiation, promotion and malignant conversion (progression) stages of carcinogenesis. Given that cumulative cancer risk increases with the

fourth power of age, and is associated with an accumulation of DNA damage, it is reasonable to investigate the potential role of oxidatively generated DNA damage in cancer.

Lesions such as 8-oxoGua are established biomarkers of oxidative stress and, coupled with their potential mutagenicity in mammalian cells, this has lead to their proposed potential as intermediate markers of a disease endpoint, such as cancer. Supportive of this proposal are the findings that GC→TA transversions, potentially derived from 8-oxoGua, have been observed *in vivo*, in the *ras* oncogene and the *p53* tumour suppressor gene in lung and liver cancer. Of course, GC→TA transversions are not unique to 8-oxoGua, whereas CC→TT substitutions, in the absence of UV light *i.e.* in internal tumours, have been identified as signature mutations for ROS [33].

Numerous studies have attempted to establish a relationship between levels of oxidatively generated DNA damage and cancer, and we have contributed to this literature. Elevated levels of damage are purported to arise as a consequence of an environment in the tumour low in antioxidant enzymes, and high in ROS generation, and/or reduced DNA repair [reviewed in [29]]. It has been widely demonstrated that the levels of free radical-induced DNA nucleobase modifications, including 8-oxoGua, are elevated in human cancerous lung tissues, compared to the cancer-free surrounding tissue [34]. Our investigations in benign tumours show that oxidatively damaged DNA may be a causative factor in cancer development. Higher levels of 8-oxoGua are observed in uterine myoma tissues, compared to corresponding tumour-free tissues [35]. Uterine myomas are some of the most common gynaecological tumours, and are monoclonal in origin, being benign tumours derived from a single mutated myometrial cell. One of the factors that may predispose to malignant transformation is the size of the tumour. We showed a positive correlation between the tumour size, the amount of 8-oxoGua [35] suggesting that higher levels of 8-oxoGua in benign tumours may itself be a risk factor for malignant transformation. Furthermore, the increased levels of modified DNA nucleobases may contribute to the genetic instability, and the metastatic potential of tumour cells in fully developed cancers.

Gackowski et al. described a unique study in which a number of markers of oxidatively modified DNA were measured in a cohort of non-small cell lung cancer patients (all of which were smokers) and two control groups (both control groups were matched to the cancer patients for age, but only one also matched for smoking status) [36]. Besides urinary excretion of 8-oxoGua and 8-oxodG, leukocyte levels of 8-oxoGua were also analysed. The level of 8-oxoGua in leukocyte DNA from cancer patients was significantly higher than in either control group. The urinary levels of 8-oxoGua and 8-oxodG were similar in both the cancer patients and the control group with similar smoking status, suggesting that the higher concentration of 8-oxoGua in lung

cancer patients' leukocytes is a result of a DNA repair defect, most likely in BER. This proposal is supported by the measurement of 8-oxoGua DNA glycosylase activity in the leukocytes of both smoker groups. Total 8-oxoGua DNA glycosylase activity was lower in the smoking cancer patients, compared to the disease-free smokers. Consistent with this, *OGG1* knockout mice are predisposed to develop lung carcinoma and 8-oxoGua accumulates in their DNA [37].

To better understand the role of oxidatively damaged in lung cancer development, three DNA damage/repair parameters in non-small cell lung cancer patients were studied: DNA 8-oxoGua levels; 8-oxoGua glycosylase activity; and 8-oxodGTP hydrolysis activity in tumour and surrounding normal lung tissue were compared [38]. Despite 8-oxoGua glycosylase activity being lower in tumour than in normal lung tissue, levels of 8-oxoGua were significantly lower in tumour than in normal lung tissue. In contrast, 8-oxodGTP hydrolysis activity was higher in the tumours, than in normal lung tissue (and three orders of magnitude higher than that of glycosylase activity). The results support the suggestion that several different components of the "GO" system interact, and contribute to the maintenance of 8-oxoGua levels in human DNA, with the greatest contributor appearing to be the removal of 8-oxodGTP from the cellular 2'-deoxyribonucleotide pool [38]. These early findings have contributed to the foundations for the recent intense interest in the enzyme(s) that remove 8-oxodGTP (and other oxidised nucleic acid precurors), as a potential therapeutic target (reviewed in [39]). This also raise the possibility that urinary 8-oxodG may be used to non-invasively give some indicator of 8-oxodGTPase activity, given that global genome nucleotide excision repair and transcription repair have been ruled out a sources of this lesion in urine [40].

Clearly, alterations in repair activity towards DNA damage have the potential to affect lesion levels, and hence outcome. Whether this of significance, in terms of predisposition to disease, remains to be established [41]. Taken together, these data suggest that, whilst the role of oxidative stress in carcinogenesis appears to be well established, the extent to which oxidatively generated DNA damage contributes has not been well defined - with a "black box" separating damage initiation (and events such a mutation, microsatellite instability, loss of heterozygosity etc) from the development of disease Apart from a few notable exceptions, such as aflatoxin-induced DNA damage, this appears to be the case for many forms of DNA damage, not just oxidatively generated DNA damage.

Although the precise role for oxidatively generated damage in cancer (or indeed many diseases) is not well defined, we propose that oxidatively damaged DNA plays an important role in malignant transformation. This is supported by the presence of multiple pathways for its repair,

clearly the cell needs to prevent damage persistence. Indeed, there appears to be no limit to the number of pathological conditions in which elevated levels of oxidatively damaged DNA have been reported [33]. Of course, the mere presence of damage is not proof of a causative link but, given the close link between ROS formation and oxidatively generated DNA damage and the importance of DNA damage and mutation in carcinogenesis, it is not a large leap of intuition to link oxidatively generated DNA lesions and cancer. What is not clear is why elevated levels of damage, in some instances, leads to cancer, but in others leads to any one of a wide range on non-malignant diseases [42]. We propose that perhaps it is not the total levels of damage that is important, but the distribution of damage. Recently methodology to map various forms of DNA damage across the genome, at varying levels of resolution was described [43,44]. We propose that approaches such as these represent a major advance for our understanding of the role of oxidatively damaged DNA in disease.

Taken together, these literature suggests that, whilst the role of oxidative stress in carcinogenesis appears well established, the extent to which oxidatively damaged DNA contributes is less well defined. Nevertheless, it appears that the DNA damage can be more closely associated with initiation events, rather than promotion, and this may be due to the potential for a multiplicity of mutagenic lesions to be formed, along with their effects on cell function [41], and, as being more recently described, perhaps epigenetic effects [45,46], see below.

3.3. Potential regulatory role of 8-oxoGua

There is no doubt that oxidative stress can lead to cell/tissue injury, via the reaction of ROS with biomolecules, and may be responsible for a variety of disparate disease processes. Recently researchers have begun to appreciate that some ROS were exploited by natural selection to act as mediators of physiological processes. Likewise, several recent reports strongly suggest that the presence of 8-oxoGua in DNA may be used as epigenetic factor rather than just inducing toxic/mutagenic damage to this macromolecule. Indeed, 8-oxoGua is nontoxic, weakly mutagenic and is readily repaired. Moreover, some experimental data demonstrate that a high background level of this modification can be tolerated without major consequences for the cell [47].

There is evidence that demonstrates that 8-oxoGua generation may regulate transcriptional activation, derived from experiments involving estrogen-responsive genes, Myc targeted genes and hypoxia-inducible genes [48-51]. It was demonstrated that exposure of cells to estrogen substantially increases 8-oxoGua in the promoter region of estrogen-responsive

genes, which in turn recruits OGG1 and topoisomerase IIβ. OGG1 generates transient nicks, which allows entry of the topoisomerase, the activity of which relaxes the DNA strand, triggering chromatin conformational changes essential for estrogen-induced transcription [48]. It has been shown that a similar mechanism is responsible for Myc-induced transcription activation [50]. It is proposed that upon binding of above mentioned transcription factors to the specific sequences, demethylating enzyme lysine-specific demethylase 1 (LSD1) is recruiting. LSD1 is a FAD-containing enzyme which is responsible for H_2O_2 production during demethylation process [52]. This H_2O_2 can cause the formation of 8-oxoGua at discrete foci and trigger a series of events, as described above, which can lead to transcriptional activation [51]. Recently it was demonstrated that generation of 8-oxoGua in G-rich regulatory elements in the promoters of several genes (VEGF, TNF-α, SIRT1) is directly linked to an increase transcription through the activation of BER pathway (reviewed in Fleming & Burrows [46].

We reported an approximately five-fold increase in 8-oxoGua in transcriptionally active nuclei compartments (euchromatin and matrix fraction) in comparison to transcriptionally silenced heterochromatin [45]. Our data suggest that the presence of 8-oxoGua in specific DNA sequences may be widely used for transcription regulation, not just restricted to above reported genes, and are in line with the hypothesis which proposes an epigenetic role for 8-oxoGua in DNA. This proposed epigenetic regulation by 8-oxoGua would be in contrast to the role of 5-mCyt, which is responsible for transcription suppression. Interestingly, in our recently published study, we demonstrated a negative correlation between background levels of 8-oxoGua and 5-mCyt in DNA [27]. The mechanisms that underly site-specific DNA modifications, chromatin changes, and transcription, are highly complex and may depend on numerous factors, such as sequence context, tissue differences or/and specific proteins assembly, to name a few [48,50]. Therefore, the context in which 8-oxoGua is present in DNA may be decisive concerning its mutagenic or epigenetic potential (see also Fleming & Burrows [46]. Although proposal for the involvement of 8-oxoGua in transcription regulation is not universally accepted, it should be remembered that the analogous process of iterative oxidation of 5-mCyt and BER, as a simple mean of demethylating DNA and activating genes, is well documented [2,53].

3.4. Steady state levels of 8-oxoGua in cellular DNA

Historically, a plethora of previous studies have quantified the level of 8-oxoGua in cellular DNA, but their results varied considerably. The first trial to standardize the analysis of 8-oxoGua in DNA was made over twenty years ago by ESCODD. After several trials, along with some standardization and refinement of methodology, consensus was achieved between HPLC-

EC and the comet assay, and the background levels of 8-oxoGua in human leukocytes was estimated to be between 0.3 and 4.2 per 10^6 dG [54], determined as the median of the values obtained from a dozen of European laboratories. These values appear to be rather accurate for a steady state, and recently reported values remain consistent with those described by the ESCODD, *e.g.* [4,27].

4. Epigenetic effects of modified DNA nucleobases

4.1. 5-Methylcytosine and their derivatives

Cyt methylation, usually at CpG dinucleotides, is one of the most important epigenetic modifications which has a profound impact on gene expression (silencing gene expression), cellular identity, and organismal fate. The reverse of DNA methylation (demethylation) is equally important to activate previously silenced genes. Although an accumulation of evidence suggests that active demethylation is possible in mammalian cells, its molecular mechanism has remained largely enigmatic [for review [2]]. The first discoveries described in two independent reports published in *Science* [55,56] demonstrated that 5-mCyt may be oxidized to 5-hmCyt in mammals and, in genomic DNA, may represent 0.003 - 0.6% of all Cyt [53]. After the "rediscovery" of 5-hmCyt in 2009, the results of a plethora of studies have confirmed the pivotal role of this modification in active DNA demethylation [2,57,58] while a role for the other derivatives (see below) is more obscure, not least due to the lack of highly sensitive methods for their detection and quantification.

The most plausible mechanisms for active 5-mCyt demethylation includes the involvement of TET proteins in oxidation of 5-mCyt to 5-hmCyt, which can be further oxidized to 5-fCyt and 5-caCyt (Figure 2). BER, acting via TDG, replaces 5-fCyt, 5-caCyt with Cyt to demethylate DNA [review in [2]]. A second scenario involves prior deamination of 5-mCyt to Thy, which generates a Gua:Thy mis-pair, and is a substrate for TDG (or some other Gua:Thy glycosylase). Here, cytidine deaminase of the AID/APOBEC family is implicated in the deamination step. It is possible that TDG may act in concert with these deaminases [59]. It is also possible that an, as yet unspecified, 5-hmCyt glycosylase is involved in its direct removal, with subsequent replacement with an unmodified Cyt [60]. 5-hmCyt could also be deaminated by AID to yield 5-hmUra which, in turn, may be removed by TDG or an enzyme from the single-strand-specific monofunctional uracil DNA glycosylase (SMUG) family (reviewed in [57]).

It is not clear why, in some instances, the TET enzymes are the major, or only, regulators of DNA demethylation. It is also not clear why, in some contexts, 5-hmCyt is the major product and in others 5-fCyt and 5-caCyt are formed *via* iterative oxidation. As mentioned above, 5-

hmCyt is a key player in the active demethylation process, with some data suggesting that it may also play a role in regulation of gene expression.

4.2. Is there a link between active demethylation and malignant transformation?

It has long been known that DNA hypomethylation occurs in many human cancers and precancerous conditions [61]. This has led to the suggestion that the hypomethylation might be responsible for enhanced genetic instability and malignant transformation. However, the mechanisms of hypomethylation are largely unknown, and hence it is unclear whether this epigenetic phenomenon is a cause or consequence of malignant transformation (for a review see [62]).

It has been found that the level of 5-hmCyt is several fold lower in DNA isolated from some solid tumours, compared to surrounding normal tissues [63]. It is unclear how or why 5-hmCyt is decreased in cancer tissues. It is possible that decreased activity of TET proteins may be responsible for this phenomenon [64], or 5-hmCyt is lost passively through replication since an inverse relationship between 5-hmCyt and 5-fCyt levels and cell proliferation is observed [65,66]. It is also possible that active DNA demethylation occurs under different conditions (e.g. chronic inflammation). It is noteworthy that both 5-fCyt and 5-caCyt have promutagenic properties and may themselves be involved in malignant transformation [67,68].

As mentioned above, it is proposed that 5-hmCyt could be deaminated by AID (or another enzyme from the APOBEC family) to yield 5-hmUra, which is then removed by TDG or SMUG. Although there are no firm experimental data which directly prove this assumption, efficient removal of 5-hmUra from the genome supports this hypothesis [69]. Interestingly 5-hmUra was originally identified as an oxidatively modified DNA nucleobase derivative (a product of thymine oxidation). However, the 5-hmUra:Ade base pair generated during this reaction is not miscoding [57,69] and does not perturb DNA structure [70]. Indeed, in some bacteriophage, 5-hmUra completely replaces Thy [71]. Moreover, the 5-hmUra:Ade base pair is poor substrate for TDG and SMUG, whereas the 5-hmUra:Gua mispair, which is formed during active demethylation, is excised with 60 times greater efficiency, and TDG does not excise 5-hmUra when paired with Ade [72]. These results suggest that there is no need for the removal of 5-hmUra paired with Ade. Therefore, the existence of 5-hmUra glycosylase activity, which very quickly and efficiently removes this modification from cellular DNA [57,59,73], suggests that the purpose of this activity is to remove 5-hmUra (specifically when paired with Gua), formed during oxidation/deamination of 5-mCyt [74].

4.3. Current knowledge of the role of 5-hmCyt in tumour development

Several recent studies show that genomic levels of 5-hmCyt are profoundly decreased in many types of human malignancies, compared to matched non-tumour tissue [75,76]. Furthermore it has been demonstrated that lower 5-hmCyt levels are an distinctive epigenetic indicator of clinical outcome, and correlates with an increased risk of neoplastic progression [75]. For example, in the case of human hepatocellular carcinoma, the level of 5-hmCyt correlates with tumour stage [77]. It is possible that a decrease in 5-hmCyt levels is due to the decreased expression of TETs. Loss-of-function mutations in *TET2* have been described in various hematological malignancies, including 7% to 10% of acute myeloid leukemia (AML) patients [discussed in [78]]. Pronier et al. demonstrated that TET2 knockdown skews human progenitor differentiation toward the myeloid lineage, giving an advantage to monocytic development at the expense of the granulocytic lineage [79]. The increased prevalence of *TET2* mutations in older patients with hematological cancers [80], together with association between *TET2* mutations and age-associated skewing in blood cells, particularly within the myeloid compartment of elderly subjects [81], further support an initiating role for *TET2* mutation in the pathogenesis of age-associated hematological cancers. These data, in combination with recent studies in murine systems, support a model where TET2 mutations in hematopoietic stem cells (HSC) confer enhanced self-renewal and clonal expansion resulting in age-related myeloid lineage bias.

TET1 and *TET3* mutations are very infrequent in hematologic malignancies, with only a few *TET1* mutations reported in chronic lymphocytic leukemia, AML, and T-cell acute lymphoblastic leukemia. Similarly, *TET3* mutations are occasionally, but very rarely, identified in peripheral T-cell lymphomas and chronic lymphocytic leukemia [reviewed in [82]]. However, recent studies indicate that *TET1* also retains a crucial regulatory role in the oncogenic transformation of hematopoietic cells. Intriguingly, *TET1* has contrasting roles in myeloid and lymphoid transformation. In mice, *TTE1* loss results in aberrant hematopoiesis characterized by an augmented repopulating capacity of HSCs, skewed differentiation toward B cell lineage and increased frequency of lymphoid-primed multipotent progenitors. TET-deficient, pre-B cells also display enhanced serial replating capacity and accumulation of DNA damage, potentially through downregulation of genes encoding components of the DNA repair pathways. Using *TET1* and *TET2* double-knockout mice, Zhao et al. showed that TET1 and TET2 are often concomitantly downregulated in acute B-lymphocytic leukemia, and that deletion of both *Tet1* and *Tet2* in mice leads to lethal B cell malignancies [83]. Interestingly, TET1 was required for *TET2*-deletion-mediated HSC dysregulation and myeloid malignancy [83]. Taken together, the above suggest

that a decrease of genomic 5-hmCyt may be diagnostic and prognostic biomarker of many cancer types.

4.4. Baseline levels of epigenetic DNA modification

There are many proteins that specifically recognize the epigenetic DNA products of active DNA demethylation. These modifications may control cell identity and, as mentioned above, play some role in tumor development [reviewed in Ficz et al. [84]]. Therefore, knowledge of their baseline level can provide important information concerning their biological relevance. The authors of several studies determined the levels of all possible intermediates of active demethylation products [4,27,85-88], but only a few of them used the gold standard technique, *i.e.* stable-isotope-dilution tandem mass spectrometry. This is the most advanced technique used for quantitative determination of a wide spectrum of endogenously generated DNA nucleobase modifications. In our laboratory, we utilize isotope-dilution automated online two-dimensional ultra-performance liquid chromatography with tandem mass spectrometry (2D-UPLC-MS/MS) [27]. This allows the direct analysis of certain DNA modifications, following digestion, with high precision, and no need for sample enrichment [85], or derivatisation [87]. Our results for brain levels of 5-hmCyt were consistent with those of previous studies [53,65,85,86]. However, we [27] and others [4] noted that all modifications were present at very low abundance, and 5-fCyt, 5-caCyt, 5-hmUra, specifically, were several-fold lower than those reported by Liu et al. [85]. All abovementioned groups included sample prepurification and concentration in offline mode between the chromatography steps and, with the exception of Liu et al. [85], used stable-isotope-dilution tandem mass spectrometry. Pfaffeneder et al. performed one-dimensional separation whereas Gackowski et al. [27] applied automated online two-dimensional separation what gave at least several times lower limits of detection. In agreement with previous studies [76,89], 5-hmCyt levels reported by Gackowski et al. were five-fold lower in colorectal carcinoma tissue in comparison with normal tissue taken from the tumour's margin. Interestingly, 5-fCyt and 5-caCyt were also lower in colon carcinoma tissue (ca. 2.5 and 3.5 fold, respectively).

It is noteworthy that in the majority of studies where 5-hmCyt, (and other epigenetic DNA modifications),were assessed, a semi-quantitative approach (immunohistochemistry) was used. The authors of these studies admitted that, although this method is easy to conduct, its ability to accurately quantify these modifications is rather poor and it may not be able to reliably determine very low levels in DNA [76], although the potential for spatial localisation of the modifications within the tissue is a strength of this approach. This kind of determination depends mostly on an

antibody affinity/specificity towards specific modification. Therefore, for precise, absolute quantification we recommend using chromatographic techniques, combined with tandem mass spectrometry, involving internal standards labelled with stable isotopes, as a "gold standard" in global genome quantification of 5-hmCyt and the other DNA epigenetic modifications [90].

5. Anticancer therapy is linked with the alternation of endogenously generated DNA nucleobase damage

5.1. Aberrant Ura incorporation into DNA

Accumulating evidence suggests that Ura incorporation into DNA contributes to cytotoxicity, resulting from the inhibition of thymidylate biosynthesis. Thymidylate biosynthesis has a long history of being an important target for several anticancer drugs [*e.g.* fluoropyrimidynes - 5-fluorouracil and 5-fluorodeoxyuridine (Figure 5), and the antifolates *e.g.* methotrexate], and is widely used as a target in the treatment of a broad range of neoplastic diseases including head and neck, breast and gastrointestinal cancers. Studies investigating the molecular mechanisms of the cytotoxic effects of these chemotherapeutics seem to suggest that cell killing is caused by a process called "thymineless death". During this process the dTTP pool is depleted and dUMP pool accumulates and is subsequently phosphorylated to dUTP by the action of kinases. As noted above, imbalance between the dTTP and dUTP pools may overwhelm cellular dUTPase activity, resulting in extensive mis-incorporation of dUTP into DNA. This will result in high levels of UDG-mediated repair [91], with the potential for AP site generation, and the increased risk of cell death *via* double strange brakes formation. Supportive of this are studies in *S. cerevisiae* strains which differed in dUTPase and UNG1 activity. All strains experienced dTTP pool depletion during antifolate treatment, but only those in which there was an accumulation of dUTP displayed severe cytotoxicity. Moreover, the cells with diminished dUTPase activity were particularly sensitive to the treatment, whereas overexpression of the dUTPase greatly decreased sensitivity to antifolate [91]. Furthermore, the results of this study demonstrated that the inactivation of Ura repair during antifolate treatment has a dramatic effect on both cell lethality, as well as a distinct checkpoint response [91]. Deletion or inhibition of *UNG1* gene product resulted in a short term enhancement of cell viability after the treatment and completion of DNA replication with incorporation of Ura instead of Thy. However, the cells which arrested in the G_2–M phase of cell cycle exhibited delayed toxicity. The authors hypothesized that Ura incorporation, instead of Thy, might be signaling the checkpoint [91].

In another study, the expression pattern of dUTPase in normal and cancerous tissues, and the association between the enzyme expression and response to 5-fluoroUra, was examined [10].

According to the authors, nuclear expression of dUTPase in cancer cells may be a prognostic marker for resistance to 5-fluorouracil in metastatic colon cancer patients [10]. This study suggests that elevated expression of nuclear dUTPase in tumour cells may protect cells from the cytotoxic effect of Ura mis-incorporation induced by inhibition of thymidylate metabolism [10]. It is also noteworthy that DNA-directed cytotoxicity of chemotherapeutic agents such as 5-fluorouracil depends not only on dTTP pool depletion, but also may be driven by the efficiency of the repair mechanisms such MMR and/or BER [92].

5.2. Anticancer therapy leads to the formation of oxidatively generated DNA damage – a possible involvement of the modified nucleobases in the development of secondary cancers

Paradoxically many of the agents used in anticancer therapy are responsible for the induction of secondary malignancies, in part through the generation of free radicals. There is a well established risk associated with developing secondary cancers after chemotherapy and radiotherapy [93,94]. Long-lived B and T lymphocytes may serve as a target cells for carcinogens including some anticancer drugs and ionizing irradiation. Some nucleobase modifications, if not repaired in the lymphocytes, could lead to mutagenesis in critical genes and ultimately to secondary cancers. Since free radical induced DNA damage may possess mutagenic properties, and may play a role in carcinogenesis, we have examined whether the modalities used in anticancer therapy are responsible for the production of typical free radical induced nucleobase modifications in the nuclear DNA of lymphocytes in cancer patients who are undergoing anticancer therapy. Ionizing radiation is one of the most commonly used therapeutic agents in cancer, with approximately half of all cancer patients receiving radiation therapy. The result of our studies demonstrate that exposure of cancer patients to therapeutic doses of ionizing radiation causes collateral nucleobase modifications in the genomic DNA of their lymphocytes (reviewed in [34]. Anthracycline derivatives have been widely used in the treatment of several types of human malignancies. The cytotoxicity of these drugs has been attributed to the inhibition of topoisomerase II, as well as the intracellular production of free radicals. Using GC-MS, Akman et al. showed that ROS production by the redox cycling of the doxorubicin quinone moiety, is responsible for DNA nucleobase modification in isolated human DNA [95]. In our work, using epirubicin (the analog of doxorubicin presenting a different configuration of the -OH group in the C-4 position of the amino-sugar moiety), we observed similar nucleobase modifications in DNA isolated from the lymphocytes of cancer patients undergoing chemotherapy [96]. The distinct pattern of these modifications suggest the involvement of $^{\bullet}OH$ in their formation. Whilst

anticancer therapy causes significant increases in DNA nucleobase modifications, there was a broad, inter-patient distribution of levels. This variation may reflect individual differences in metabolism and repair capacity, and/or genetic predisposition [34]. For the majority of patients, the levels of the nucleobase products returned to pre-exposure levels 24 h after treatment. Similar results were observed in the lymphocyte DNA of the patients who were undergoing radiotherapy. This decrease in the level of nucleobase products may be an indication of their repair which, in the case of some patients whose the level of certain modifications remained high, suggest an impairment of the DNA repair capacities [96,97].

5.3. Azacytidines as drugs with epigenetic effects

The azacytidines are analogues of cytidine (Figure 5). Those currently used in the clinic are: 5-azacytidine (azacytidine) and 5-aza-2'-deoxycytidine (decitabine), and are known inhibitors of DNA methylation [98]. They are used for the treatment of AML and myelodysplastic syndrome. When given at low doses (2 mg/m^2/day for 7 days), 5-aza-2'-deoxycytidine inhibited promoter-specific as well as global DNA methylation [99]. Using hepatocellular cell lines, it was shown that 5-azacytidine can also influence DNA methylation status, triggering an active demethylation pathway through activation of TET2 and TET3 proteins [100]. More recently it was demonstrated that combination treatment of AML recipient mice with 5-azacytidine and clinical grade drug AG-221, which inhibits mutant isocitrate dehydrogenase and reduce 2-hydroxyglutarate, a potent inhibitor of TETs protein, can effectively revers hypermethylation (reviewed in [101]).

6. Conclusions

Endogenously generated DNA modifications are a consequence of normal cell metabolism, but little is known about their levels, in terms of what might be considered a "normal", or a reference range, what changes may reflect a potential decrease or increase in cancer risk, and what/how dietary and environmental factors may modulate these levels. Recently, some researchers have started to speculate that at least some DNA nucleobase modifications might be drivers in natural selection, acting as genetic or epigenetic mediators or modulators of physiological processes. Therefore, the assumption that the processes which lead to their production should be avoided at any cost, now appears to be misguided. Intriguingly, all endogenously generated, modified nucleobases seem to play important cellular regulatory roles, for example:

(i) Mutagenesis does not seem to be the only effect of 8-oxoGua. While the other effects of this compound are still not completely understood, but available evidence suggests that 8-oxoGua may also affect expression of several genes *via* chromatin relaxation [48], or the binding of transcription factors to gene promotors. Furthermore, the cell type-specific DNA level of this modification is suggested to be essential for cellular physiology [49,102].

(ii) The immune system of higher organisms has a strategy to use intentionally generated Ura for *Ig* gene diversification. Also, we have suggested [9] that Ura present in DNA (in Ura:Gua as well as Ura:Ade mis-pairs) may also perform an, albeit undefined, regulatory role.

(iii) Oxidatively generated derivatives of 5-mCyt play an important role in active process of DNA demethylation and may have specific regulatory functions [103].

We note the requirement for reliable identification and quantification of DNA adducts/modifications, whether or not endogenously-derived, and that can aid informing on the mechanism of their action and biological relevance. Clinical application of such measurements needs to be preceded by full assay validation and a better understanding of the exact role played by DNA modifications in the disease pathogenesis. Once these prerequisites are satisfied, DNA modification measurements may be helpful as a clinical parameter for treatment monitoring, risk group identification and development of prevention strategies. Consequently, this knowledge could be used for monitoring cancers, and other diseases related to oxidative stress, aberrant metabolism and environmental exposure.

The above clearly demonstrates that, for DNA, the terms “damage” and “modification” have different implications for the cell, but what dictates whether the presence of an altered nucleobase is defined as damage, or a lesion, or (more benignly) a modification? Ura, mis-incorporated into DNA, is not damage per se, it is not even a modified nucleobase, unlike deaminated Cyt. It is inappropriate and, as a result of its presence, the DNA has been modified. 8-oxoGua, however, is both a form of damage, and a modification of Gua, and may have both potentially benign and detrimental consequences for the cell. Classically, endogenous modifications are viewed to be less detrimental to the cell, after all why would a consequence of normal metabolism be detrimental to the cell? However, when ROS generation from mitochondrial respiration, or redox cycling xenobiotics is considered, the situation becomes less clear cut. We propose that these examples should prompt us to rethink the definitions of damage etc, not least since applying the most appropriate term seems to be depend upon the local context in a DNA sequence, and possible downstream effects.

It is also puzzling why potentially mutagenic DNA damage, which occurs at a similar level in most tissues, may lead to cancers in some, or non-malignant disease in others? What

process determines the outcome? Perhaps the epigenetic DNA nucleobase modifications e.g. 5-hmCyt and the higher order products of TETs, play a role, as these possess considerable inter-tissue differences in the levels. We suggest that perhaps it is not simply levels of damage, but location of damage that is the key factor, requiring a new generation of techniques, evaluating DNA at a 2'-deoxyribonucleotide resolution, to better understand the functional consequences of modified DNA.

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Figure legends

Figure 1.

Structure and nomenclature of endogenously generated DNA nucleobases modifications, 2'-deoxyribonucleosides and 2'-deoxyribonucleotides.

Figure 2

Cytosine methylation and active demethylation pathway. 5-mCyt, one of the most important epigenetic modifications which has a profound impact on gene repression cellular identity and organismal fate, is formed in DNA methyltransferase (DNMT) catalysed reaction. The most plausible mechanisms of active 5-mCyt demethylation include involvement of ten-eleven translocation (TET) proteins in oxidation of 5-mCyt to form 5-hmCyt which can be further oxidized to 5-formylcytosine (5-fCyt) and 5-carboxylcytosine (5-caCyt). Then BER pathway is active by the involvement of TDG glycosylase to replace the above-described nucleobase modifications (5-fCyt, 5-caCyt) with cytosine to demethylate DNA. 5-hmUra may also be generated by TET enzymes from thymine as well as to play an important role in active DNA demethylation when is generated via deamination pathway.

Figure 3

Generation of Ura and 8-oxoGua in nuclear and mitochondrial DNA, and the multiple mechanisms for preventing their persistence. Prevention of mis-incorporation: dUTPase maintains the critical ratio between the dUTP and dTTP pools, whilst also forming dUMP as a precursor for dTTP synthesis; 8-oxodGTPases hydrolyse oxidized 8-oxodGTP to 8-oxodGMP. *BER*: Ura glycosylases and MMR remove Ura from DNA; enzymes such as hOGG1 remove 8-oxoGua, and hMYH removes mis-matched native nucleobases opposite 8-oxoGua, formed following mis-incorporation of 8-oxodGTP. Although not illustrated here, separate pre-cursor pools feed the nuclear and mitochondrial genomes.

Figure 4

Mechanisms of mutations generated as a consequence of cytosine deamination. Processing of Ura, formed as a result of AID activity, recruits UNG2 and MSH2-MSH6 from the BER and mismatch repair (MMR) pathways, respectively. There are two distinct phases of SHM; the first depends on the activity of AID, and the second phase depends on the mutagenicity of the error

prone repair of AID-induced substitutions. Phase one occurs when UNG2 removes Ura, formed after AID, and creates an abasic site (which has no coding potential) then, in phase two, this abasic site can be filled in with any of the four nucleobases. Phase two depends mostly on the error-prone MMR, which recognizes an Ura:Gua mispair. A section of the Ura-containing strand is removed, and monoubiquinylated PCNA attracts members of a family of low-fidelity translesional DNA polymerases which contribute to nucleotide substitutions at an unprecedented level.

Figure 5

Nucleosides analogues used as anticancer drugs which may possess ”epigenetic” mechanisms of action

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