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Mutagenic potential of hypoxanthine in live human cells

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Abstract

Hypoxanthine (Hx) is a major DNA lesion generated by deamination of adenine during chronic inflammatory conditions, which is an underlying cause of various diseases including cancer of colon, liver, pancreas, bladder and stomach. There is evidence that deamination of DNA bases induces mutations, but no study has directly linked Hx accumulation to mutagenesis and strand-specific mutations yet in human cells.

Using a site-specific mutagenesis approach, we report the first direct evidence of mutation potential and pattern of Hx in live human cells. We investigated Hx-induced mutations in human nonmalignant HEK293 and cancer HCT116 cell lines and found that Hx is mutagenic in both HEK293 and HCT116 cell lines. There is a strand bias for Hx-mediated mutations in both the cell lines; the Hx in lagging strand is more mutagenic than in leading strand. There is also some difference in cell types regarding the strand bias for mutation types; HEK293 cells showed largely deletion (>80%) mutations in both leading and lagging strand and the rest were insertions and A:T→G:C transition mutations in leading and lagging strands, respectively, whereas in HCT116 cells we observed 60% A:T→G:C transition mutations in the leading strand and 100% deletions in the lagging strand. Overall, Hx is a highly mutagenic lesion capable of generating A:T→G:C transitions and large deletions with a significant variation in leading and lagging strands in human cells. In recent meta-analysis study A→G (T→C) mutations were found to be a prominent signature in a variety of cancers, including a majority types that are induced by inflammation. The deletions are known to be a major cause of copy-number variations or CNVs, which is a major underlying cause of many human diseases including mental illness, developmental disorders and cancer. Thus, Hx, a major DNA lesion induced by different deamination mechanisms, has potential to initiate inflammation-driven carcinogenesis in addition to various human pathophysiological consequences.

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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Keywords

DNA damage; cancer; hypoxanthine; inosine; inflammation; MPG; mutation; OGG1; reactive oxygen and nitrogen species; base excision repair; site-specific mutagenesis; deamination damage

1. Introduction

Hypoxanthine (Hx, the base component of 2'-deoxyinosine, dI) is a DNA lesion induced mainly by endogenous agents during chronic inflammation. Hx can be formed by different deamination mechanisms during inflammation. This includes simple DNA hydrolysis and reaction with nitrous anhydride (N₂O₃), a genotoxic derivative of nitric oxide [1–4]. However, nitric oxide-induced deamination of adenine to form Hx is not strongly supported [5]. Under inflammatory conditions in addition to deamination of adenine and formation of Hx (dI; Fig. 1A), guanine, cytosine and 5-methylcytosine are also deaminated to form xanthine, uracil and thymine, respectively [2,4]. Moreover, bases deaminate not only within the DNA molecule, but also as nucleoside triphosphates, precursors of DNA. Then a noncanonical nucleotide, such as dITP, is incorporated into the newly synthesized DNA, albeit less efficiently compared to dATP [6].

The level of Hx is detectable in animals (~5.7 lesions per 10⁷ nucleotides), but it increases significantly during inflammation in infection-induced colitis and colon cancer models [7]. In general, DNA damage levels are known to be altered in cells by DNA repair pathways or through the replication process, which often convert DNA lesions to mutations [8–10].

Hx is known to be repaired efficiently by the base excision repair pathway. A monofunctional DNA glycosylase, N-methylpurine DNA glycosylase (MPG) recognizes Hx and efficiently excises the Hx base leaving a cytotoxic abasic site (AP-site). AP endonuclease 1 (APE1) nicks the DNA at AP-site leaving a 5'-deoxyribose phosphate (dRP) moiety. A repair polymerase, DNA polymerase β fills in the nucleotide gap and removes the dRP. Finally, a DNA ligase seals the nick and completes Hx repair [10,11].

In several studies with *E. coli*, human cell line and transgenic mouse model, NO has been shown to have mutagenic activity under inflammatory conditions [12,13]. However, those studies did not examine Hx or other deaminated bases or direct relationship of deaminated bases with mutations. Moreover, no study has linked Hx accumulation to mutagenesis and strand-specific mutations in human cells. We used a site-specific mutagenesis approach to investigate Hx-induced mutations in the replication context of both leading and lagging strands in human cells.

2. Materials and methods

2.1 Construct preparation and single-strand DNA (ssDNA) isolation

We constructed a replicating shuttle vector as previously described by Yoon et al [14]. Essentially, the pBluescript II (KS⁺/SK⁻)-SV40 (pBSII(KS⁺/SK⁻)-SV40) was prepared by cloning a DNA fragment (700 bp) containing SV40 replication origin sequence effectively in two opposite orientations with respect to the placement of Hx and 8-oxo-G at the EcoRI site

within the multiple cloning site (MCS). The SV40 origin sequence was amplified from pSP189 using primers containing AflIII and SapI restriction sites and cloned in either pBSII (KS⁺) or pBSII (SK⁻) at AflIII and SapI sites. The phage suspension was prepared following a standard method [15]. Single-stranded pBSII(KS⁺/SK⁻)-SV40 DNA was isolated from the phage suspension using the Qiaprep Spin M13 kit and following manufacturer's protocol (Qiagen, Gaithersburg, MD).

Leading orientation was established when Hx was placed in the MCS (see below for method of Hx-containing DNA preparation) by primer extension on the KS⁺ single stranded DNA (sense strand) so that the SV40ori is located on the 3' side of Hx. During DNA synthesis, Hx is encountered by the polymerase while synthesizing the leading strand. Similarly, lagging orientation was established when Hx was placed in the MCS by primer extension on the SK⁻ single stranded DNA (antisense strand) so that the SV40ori is on the 5' side of Hx. Then during DNA synthesis the polymerase encounters Hx while synthesizing the lagging strand (Fig. 1B and C).

2.2 Hx- or 8-oxo-G- pBSII(KS⁺/SK⁻)-SV40 in vitro construct preparation

The modification (Hx or 8-oxo-G) was placed at the EcoRI site of the pBSII(KS⁺/SK⁻)-SV40 construct as described previously for 1, N⁶ ethenoadenine constructs [10]. Briefly, the preparation included four main steps: phosphorylation of two separate damage-containing primer oligonucleotides (5'-CCGGGCTGCAGXYATTCGATATCAA-3' where X=8-oxo-G or Y=Hx and the underline refers to EcoRI site) or the undamaged primer oligonucleotide (5'-CCGGGCTGCAGGAATTCGATATCAA-3' where the underline refers to EcoRI site), annealing of the oligonucleotides to the ssDNA, and the primer extension reaction and ligation. Then plasmid DNA was recovered using Qiaquick PCR Purification kit (Qiagen, Gaithersburg, MD). Finally, all the modified and undamaged plasmid DNA constructs were *in vitro* methylated with Dam Methylase (New England Biolabs, MA) following manufacturer's conditions. The methylated plasmid DNA was further purified using Qiaquick PCR Purification kit (Qiagen, Gaithersburg, MD).

2.3 Confirmation for the presence of Hx- or 8-oxo-G and methylation in the pBSII(KS⁺/SK⁻)-SV40 constructs

We verified for the presence of 8-oxo-G by digestion of plasmid DNA with 60 nM 8-oxoguanine DNA glycosylase (OGG1) and 100 nM APE1 via glycosylase activity assay. OGG1 and APE1 were expressed and purified previously [16]. In a similar manner, a glycosylase activity assay containing 60 nM MPG and 100 nM APE1 was utilized to verify the presence of Hx in the leading and lagging strands of pBSII-sv40ori DNA. MPG was expressed and purified previously [17]. We incubated 200 ng of 8-oxo-G- or Hx- containing pBSII-sv40 plasmids with OGG1 or MPG cocktails for 1 hr at 37°C in a reaction volume of 20 µL containing 25 mM HEPES-KOH (pH7.9), 0.5 mM DTT, 10 µg/ml nuclease-free bovine serum albumin, 150 mM NaCl and 10% glycerol. We then verified that the plasmids were nicked for the presence of 8-oxo-G and Hx by agarose gel electrophoresis. DNA methylation was tested by transforming the methylated plasmid DNA with or without DpnI (New England Biolabs, MA) digestion into XL-1 Blue followed by colony formation. The results showed that there is very few or no colony formation after DpnI digestion of

undamaged or Hx/8-oxo-G-containing plasmids (data not shown). This ensures that DNA methylation and DpnI digestion were complete. To address the possibility of contaminating hemimethylated DNA resulting from semi-conservative DNA replication in human cells and is fairly resistant to DpnI digestion [18,19], we digested hemimethylated control/Hx/8-oxo-G containing plasmids with and without DpnI. DpnI treatment resulted in very few or no colony formation (data not shown). This again ensures that our DpnI digestion condition also eliminates hemimethylated DNA. We performed EcoRI (New England Biolabs, MA) digestion followed by XL-1 Blue colony formation. EcoRI digestion of undamaged plasmid showed no colonies, whereas the same digestion of Hx/8-oxo-G-containing plasmids showed similar colony formation as the undigested plasmids (data not shown). The EcoRI digestion results ensure that both lesions are present in the plasmids and inhibit EcoRI digestion. Thus, the mutation results from human cells using site-specific mutagenesis assay (described below) are not affected by any remaining parental plasmids.

2.4 Site-specific mutagenesis assay

The damaged (Hx and 8-oxo-G) or the undamaged constructs, after methylation, and the SV40 T antigen expression vector were co-transfected into HEK293 and HCT116 cells with Lipofectamine 2000 (Invitrogen) following manufacturer's protocol and as described previously [10]. Forty eight hours post-transfection, the cells were harvested and the plasmids were recovered using Qiagen Miniprep kit (Qiagen, Gaithersburg, MD). Retrieved plasmid DNA was digested with DpnI (New England Biolabs, MA) and EcoRI (New England Biolabs, MA) or DpnI only and the digested plasmid DNA was then transformed by electroporation into XL1-Blue cells. Colonies were manually counted and mutation frequency was calculated as described previously [10]. Briefly, the mutation frequency was calculated by dividing the mutant colonies (DpnI and EcoRI-digested) by total number of colonies (only DpnI digested). DpnI digestion eliminated the parental methylated Hx/8-oxo-G-containing or undamaged DNA, leaving only progenies, which are Hx-or 8-oxo-G-free, but may have incurred mutations or wild type sequence at Hx or 8-oxo-G site. The colonies formed without EcoRI digestion provided the measure of total number of plasmid DNA copies, and the colonies that were formed after EcoRI digestion afforded the measure of copy numbers of mutant plasmid DNA as they are only able to form colonies because of resistance to EcoRI digestion. To validate this resistance, 30–40 of these colonies were tested by a second EcoRI digestion followed by agarose gel electrophoresis. However, we observed only 2–3% of colonies containing WT DNA that had escaped the initial EcoRI digestion. Therefore, those colonies did not significantly affect the overall mutation frequency reported. Similarly, the efficiency of progeny recovery was measured by analyzing the XL1-Blue colonies transformed with DpnI digested and undigested plasmid DNA recovered from human cells. Mutation patterns were analyzed by examining mutations and deletions in the EcoRI restriction site via Sanger sequencing provided by GeneWiz (Germantown, MD).

2.5 Data analysis, statistics and quantifications

Data are presented as the mean $\pm$ SE of at least 3 independent experiments and were analyzed via one-way ANOVA followed by Tukey's multiple comparison post hoc test for undamaged and Hx containing DNA studied in HEK293 and HCT116 cells, and unpaired t-test with

Welch's correction for unequal variance for undamaged and 8-oxo-G containing DNA studied in HCT116 cells. All statistical analyses were performed, and all graphs generated, using GraphPad Prism 7.0 software (GraphPad).

3. Results

3.1 Site-specific mutagenesis assay verification and validation

We have recently reported an in-cell site-specific mutagenesis assay, but utilized only for mutation frequency measurement [10]. Here we verified our new assay for both mutation frequency and mutation pattern analyses for a well-characterized mutagenic DNA adduct, 8-oxoguanine (8-oxo-G). We incorporated a single 8-oxo-dG residue within the EcoRI site of a replicating plasmid DNA in the leading strand as previously described [10]. We then verified the presence of 8-oxo-G by treating the DNA with OGG1 and APE1, which specifically removes 8-oxo-G and nicks the DNA at AP-site. Although OGG1 is a bifunctional DNA glycosylase, it leaves a substantial amount of AP-sites [20]. To ensure complete cleavage of AP-sites we included APE1 in the reaction. After treatment the covalently closed circular (CCC) plasmid DNA containing 8-oxo-G was almost completely converted to nicked DNA, while an insignificant amount of CCC undamaged DNA showed conversion to nicked form (Fig. 2A). This confirms the presence of 8-oxo-G in the plasmid DNA. We then transfected the 8-oxo-G containing pBSII-sv40ori plasmid DNA into HCT116 cells and tested the efficiency of progeny recovery. The number of progeny per 100 retrieved molecules of 8-oxo-G containing plasmid was similar to that of control undamaged plasmid (Fig. 2B). The mutation frequency of 8-oxo-G was, however, significantly higher with cells transfected with 8-oxo-G containing pBS-sv40ori (leading) plasmid compared to control undamaged plasmid (Fig 2C). This finding is consistent with the previously published results [21], and thus, which validated our methodology to examine Hx mutagenicity in human cells. Additionally, we characterized the distribution of the mutations, finding largely GC→TA transversion mutations (55.6%; Fig 2D), which was consistent with the results of others [21].

3.2 Hx construct preparation and verification

Similar to 8-oxo-dG, we incorporated a single deoxyinosine (Hx is the base) within the EcoRI site of the replicating plasmid DNAs, pBSII-sv40ori, in both leading and lagging strand in separate constructs. Then we verified the presence of Hx by treating the DNA with MPG and APE1, which specifically removes Hx and nicks the DNA at AP-site, respectively. After treatment the CCC plasmid DNA containing Hx was almost completely converted to nicked DNA, while an insignificant amount of CCC undamaged DNA showed conversion to nicked form (Fig. 3A). This confirms the presence of Hx in the replicating constructs.

3.3 Hx-induced mutations in human nonmalignant HEK293 cells

To explore the mutagenic potential of Hx in human cells, we examined the effect of this adduct in a human nonmalignant HEK293 cell line. We and others have extensively used HEK293 cells for site-specific mutagenesis of various DNA adducts [10,22]. As previously described [23], we also considered DNA synthesis mechanisms and the effects it may have on Hx-induced mutation on both the leading and lagging strand of DNA, and transfected these constructs separately in HEK293 cells.

Replication efficiency for HEK293 cells that contained Hx and undamaged pBSII-sv40ori DNA was established to compare the plasmid's effect on its replication. There were no significant differences in progeny recovery efficiencies between leading and lagging strands (Fig. 3B), which is consistent with previous findings that rates of replication fork movement are similar in both directions from the SV40 origin [24,25]. Next, mutation frequency was examined, and we found that DNA containing Hx in the leading strand had a mutation frequency of 1.06%, 2.7-fold more than the undamaged DNA (0.39%, $p<0.0162$). The DNA containing Hx in the lagging strand showed a 2.8% mutation frequency, which is 7.3-fold more than undamaged DNA (0.38%, $p<0.0001$) (Fig. 3C and Table 1).

Of the mutations that we observed in HEK293 cells, undamaged DNA generated 100% deletions, whereas DNA containing Hx in the leading strand produced 11.1% insertions, with the remaining 88.9% deletions and no substitutions at Hx site. To our knowledge, this is the first time insertions have been directly attributed to the presence of Hx in leading strand DNA (Fig. 3D). In the undamaged DNA we observed 93.8% deletions, with the remaining 6.2% AT→GC transition mutations. DNA containing Hx in the lagging strand, however, showed 84.6% deletions and 15.4% AT→GC transition mutations (Fig. 3D).

We noted that the leading strand containing no adduct or Hx did not show any transition or transversion mutations, while interestingly, the lagging strand containing either Hx or no adduct exhibited transition mutations, albeit Hx had much more than the no adduct control. Furthermore, only the leading strand containing Hx led to insertion mutations.

3.4 Hx-induced mutations in human HCT 116 cancer cells

We also tested the mutagenic potential of Hx in a human cancer cell line, HCT116. We and others have also extensively used HCT116 cells for site-specific mutagenesis of various DNA damage [10,22]. We again considered DNA synthesis mechanisms and the effects it may have on Hx-induced mutation on both the leading and lagging strand of DNA. HCT116 was transfected with pBluescript II-sv40ori containing plasmids that had been modified to contain a Hx modification in the EcoRI restriction site, in both the leading and lagging strand DNA conformations. In the HCT116 cancer cells, we did not observe any significant difference in progeny recovery efficiency for leading and lagging strands of undamaged and Hx-DNA (Fig. 4A). Next, we examined mutation frequency and observed that Hx on the leading strand of DNA resulted in a 1.5% mutation frequency which is 6.0-fold more than the undamaged leading strand DNA (Fig. 4B; $p=0.0202$ and Table 1). The Hx on the lagging strand of DNA resulted in a 2.8% mutation frequency, which is 5.6-fold more than the undamaged lagging strand DNA (Fig 4B; $p=0.0007$).

Interestingly, of the mutations observed in HCT116 cells, the undamaged leading strand DNA generated 100% deletions compared with 40% deletions and 60% A:T→G:C transition mutations seen in the leading strand with Hx. However, in the lagging strand DNA with both undamaged and Hx DNA, we observed 100% deletion mutations, albeit there was a 5-fold increase with Hx compared to undamaged DNA (Fig. 4C). These are essentially large deletions (200 nucleotides). Previous studies with pS189/*SupF* plasmid system also showed significant fraction of large deletions for undamaged DNA [26,27]. Furthermore, the spontaneous mutation frequency of the undamaged DNA in our study (0.01–0.47%) is

comparable to those previous studies by others (0.02–0.2%;[27,28]). Thus, the large deletions we are observing are not due to the difference in plasmid systems used in this study and in other laboratories, rather caused by the DNA damage.

Notably, we found that Hx when placed on lagging strand is more mutagenic than the same Hx in the leading strand in both the cell lines; Hx in the lagging strand is 2.6-fold (Fig. 3C; $p<0.0001$) and 1.8-fold (Fig 4B; $p=0.0226$) more mutagenic than the Hx in leading strand in HEK293 and HCT116 cells, respectively.

4. Discussion

We validated our previously developed site-specific mutagenesis assay[10] for both mutation frequency and mutation pattern analyses for a well-characterized mutagenic DNA adduct, 8-oxoguanine (8-oxo-G). Using this method we report the first direct evidence of mutation potential and pattern of Hx in live human cells. We investigated Hx-induced mutations in human nonmalignant HEK293 and cancer HCT116 cell lines, and found that Hx is mutagenic in both HEK293 and HCT116 cell lines, despite the fact that a part of the lesion-containing plasmids might be repaired before their replication resulting in decreased mutation frequency. There is a strand bias for Hx-mediated mutations in both the cell lines; the Hx in lagging strand is more mutagenic than in leading strand. There is some difference in cell types regarding the strand bias for mutation types. HEK293 cells showed mostly deletion (>80%) mutations in both leading and lagging strands and the remaining mutations were insertions and A:T→G:C transition mutations in leading and lagging strands, respectively. In HCT116 cells we observed 60% A:T→G:C transition mutations in the leading strand and 100% deletions in the lagging strand. The cell type difference for the mutation spectra seems interesting. Compared to nonmalignant human kidney HEK293 cells, malignant colon cell line HCT116 cells have a large number of gene mutations including over 80 oncogenes and some DNA by-pass polymerase family genes (Cancer Gene Census and Cell Line Project v80, COSMIC, Sanger, UK). This may have caused different mutation spectra in HEK293 versus HCT116 cells.

NO has been shown to be mutagenic in human TK6 cells[29], animals bearing tumors [12], and also in other human and *E. coli* cells [13]. Routledge et al showed 61% and 75% A:T→G:C transition mutations in human Ad293 cells and *E. coli*, respectively [13]. They treated SupF-containing shuttle vector with NO and analyzed the global mutation spectrum on SupF gene, but neither Hx formation on the DNA nor the strand specificity was tested. In fact, the mutation spectrum by NO can be hardly determined due to the presence of diverse DNA adducts generated by NO-produced species and its mutagenic diversity [30–32]. Therefore, signatory and quantitative mutational potential of Hx must be understood to explore its role in inflammation-driven carcinogenesis. In regard to base substitution we only observed A:T→G:C transition mutation and this is consistent with an *in vitro* study with purified human DNA polymerases by Yasui et al. Pol α , Pol η and Pol κ incorporated preferentially dCMP (55–83%) opposite the hypoxanthine lesion, and no blockage was detected [33]. It is possible that Hx might be excised by MPG and leave AP-site due to incomplete repair by the BER pathway. The AP-site, instead of the parental lesion Hx, would induce mutations, and follow rigidly the ‘A-rule’ [34]. In that manner, we would

expect A→T transversion mutation since we incorporated Hx in place of adenine in the EcoRI site. However, we observed only A→G transition mutation in both HEK293 and HCT116 cells and therefore, the substitution mutation, A→G, was most likely generated from the parental lesion, Hx. In addition to substitution mutation, we observed a significant fraction of large deletion mutations with Hx in intact human cells (Figs. 3D and 4C). Hx is a simple deamination modification of adenine and has a planar structure like purines, and thus, one can presume that Hx may not induce deletion mutations. In fact, a collection of studies on various DNA bulky lesions such as Gh, Sp, Ia, Iz, Oz indicated only substitution mutations [35–40]. However, those studies used either only *in vitro* primer extension type experiments with few purified DNA polymerases [35–38] or computer simulation-based predictions of mutagenic behavior of lesions in biological contexts [39,40]. Importantly, none of the studies were tested in live cells, and thus missed the whole replication machinery and mechanisms necessary for inducing different mutations including the replication blocks. Additionally, that *in vitro* study with Hx used a short oligonucleotide template-primer system [33], which does not allow probable mechanisms such as replication fork stall involving Polδ/ε and template switching for generation of large deletions [41].

We also noted insertion mutations on the leading Hx-containing strand in HEK293 cells. Insertion mutations may form during trans-lesion synthesis (TLS) of Hx by DNA polymerases during replication, specifically when there has been a replication fork stall [42,43]. The Y-family of DNA polymerases are error prone, and known to cause indels (including –1 and +1) when replicating past an AP-site, which may be formed via excision of Hx from the DNA without completing the BER pathway [44]. The insertion mutations that we observed on leading strands in HEK293 cells have an extra adenine at the EcoRI site: G(HX)ATTC→GAAATTC. +1 frameshift mutations may be preferential to DNA fork collapse, or large scale deletions as we observed on lagging strand in HEK293 cells, and in HCT116 leading and lagging strands [45]. Further analysis on Y-family DNA polymerases and Hx by-pass will need to be performed on leading and lagging strands to understand these intricate mechanisms.

We noticed strand bias for Hx-mediated mutations in both HEK293 and HCT116 cell lines with the Hx in lagging strand being 2 fold more mutagenic than in leading strand. Strand specificity of mutations was also observed for other DNA damage such as psoralen monoadducts in human cell extracts by Thomas et al [46]. They observed a 3-fold difference in overall error frequency per detectable site replicated in cell extracts, with lagging-strand replication being more erroneous [46]. Our results with Hx are somewhat similar to those with psoralen monoadducts. Although a new study showed that Polδ is responsible for both leading and lagging strand synthesis during normal replication [47], under DNA damage stress conditions, other DNA polymerases including Polε and lesion by-pass polymerases, proofreading exonucleases, accessory proteins and/or the architecture of the replication fork may have significant differential effect on replication of leading and lagging strands [47–50]. These possibilities may be tested in the future to understand the intricate mechanism of strand bias for Hx mutagenesis.

Although the overall A:T→G:C mutation was thought to be rare in human cancer cells, the same mutation in a particular sequence context, a purine 5' to the mutated A, is more

frequent in p53 gene of colorectal tumors, lymphomas and leukaemias [51–54]. However, in a recent meta-analysis study that included 5 million somatic mutations across 7,000 cancers, Alexandrov *et. al.* found that A→G (T→C) mutations are a prominent signature in a variety of cancers, chiefly of the stomach and liver, but also found in lymphomas, gliomas, kidney cancer, lung (small and adenocarcinoma), and pancreatic cancer [55]. This finding correlates with our observations, that Hx is formed in an environment enriched with reactive nitrogen and oxygen species, like those found in the tumor microenvironment, and that its formation leads to A:T→G:C mutation signature.

While dITP incorporation in DNA has been shown to be nonmutagenic in *E. coli* [56], in a study with human and mouse cells Yoneshima *et. al.* demonstrated that deoxyinosine (dI) causes genomic instability, and growth arrest [6]. They found that inactivating inosine triphosphatase (ITPA), an enzyme responsible for hydrolyzing dITP to dATP, allows for the unhydrolyzed free dITP to accumulate in the nucleotide pool and be incorporated into DNA, via the DNA polymerases. In fact, ITPA KO in mice leads to growth retardation and cardiac complications, leading to death before weaning [57]. Yoneshima *et. al.* predicted that dITP may cause the DNA polymerase to stall during DNA synthesis, and thus cause a replication fork collapse [6].

Repeated proofreading by the DNA polymerase is a candidate mechanism for polymerase stalling. In fact, proofreading-deficient Deep Vent polymerase (Exo–), but not proofreading-proficient Deep Vent (Exo+), could amplify DNA using Hx-containing primers. This suggests that the proofreading function of some DNA polymerases may recognize a base pair of hypoxanthine and a normal base as a mismatch pair and initiate proofreading [58]. Moreover, it is possible that AP-site accumulation due to incomplete repair by BER pathway may lead to deletion mutations. A full investigation into these mechanisms of Hx-induced deletion mutations, including the leading vs lagging strand differences in deletion frequency, presents new and interesting challenges because of the novelty of the deletion induced by Hx, which is considered a simple deamination modification of a base, and such an investigation deserves its own detailed study in the future.

In this study we provided the first direct evidence of mutation potential and pattern of Hx in intact human cells. Overall, Hx is a highly mutagenic lesion capable of generating A:T→G:C transitions and large deletions with a significant variation in leading and lagging strands. Both deletions and duplications are known to be a major cause of copy-number variations or CNVs, which are major underlying causes of many diseases including mental illness, developmental disorders and cancer [41]. Thus, Hx, a major DNA lesion induced by NO, has potential in initiating inflammation-driven carcinogenesis in addition to various human pathophysiological consequences.

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Abbreviations

Hx Hypoxanthine

8-oxoG 8-oxoguanine

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Highlights

- First report of direct evidence of mutation potential and pattern of Hx in live human cells.
- Hx generates A:T→G:C transitions and large deletions.
- Hx-induced mutations vary in leading and lagging strands.
- Recent meta-analysis study showed A→G (T→C) mutations in a variety of cancers.

Fig. 1. Strategy for testing Hx-induced mutations on the leading or lagging strand DNA template of a duplex plasmid

A) Structures of deoxyadenosine (dA) and deoxyinosine (dI). B) The SV40-containing replicating shuttle vector used for the construction of plasmid DNA containing a site-specific Hx lesion. C) The target sequence containing Hx lesion at EcoRI site in the leading strand (pBSII(KS⁺)-SV40) and the lagging strand (pBSII(KS⁻)-SV40) are shown with respect to SV40 origin.

Fig. 2. 8-oxo-G-induced mutations in HCT116 cells

A) OGG1 and APE1 digestion of undamaged and 8-oxo-G containing pBSII-sv40ori plasmids. Upon treatment with OGG1 and APE1, 8-oxo-G-containing CCC DNA is converted to nicked DNA. See Materials and methods for details. B) Replication efficiency of undamaged and 8-oxo-G-containing plasmid DNA. C) 8-oxo-G-induced mutation frequency. D) 8-oxo-G-induced mutation distributions. CCC: covalently closed circular DNA; 8-oxo-G: 7,8-Dihydro-8-oxoguanine. * $p=0.00342$.

Fig. 3. Hx-induced mutations in HEK 293 cells

A) MPG and APE1 digestion of undamaged and Hx-containing pBSII-sv40ori plasmids. Upon treatment with MPG and APE1, Hx-containing CCC DNA is converted to nicked DNA. See Materials and methods for details. B) Replication efficiency of undamaged and Hx-containing plasmid DNA. C) Hx-induced mutation frequency. D) Hx-induced mutation distributions. CCC: covalently closed circular DNA. *p 0.02, ****p<0.0001.

Fig. 4. Hx-induced mutations in HCT116 cells

A) Replication efficiency of undamaged and Hx-containing plasmid DNA. B) Hx-induced mutation frequency. C) Hx-induced mutation distributions. * $p=0.02$, *** $p=0.0007$.

Table 1

Hx-induced mutations on leading and lagging strands in HEK293 and HCT116 cells

Cell Line	Strand	Lesion	Colonies Analyzed	^a Mutation Freq.
HEK293	Leading	Undamaged	2370	0.39
	Leading	Hx	5020	1.06
	Lagging	Undamaged	56100	0.38
	Lagging	Hx	6298	2.79
HCT116	Leading	Undamaged	2363	0.25
	Leading	Hx	2866	1.49
	Lagging	Undamaged	14840	0.47
	Lagging	Hx	18660	2.8

^a Mutation frequency (number of mutants/100 colonies analyzed)