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► **To cite this version:**

Eliette Touati. When bacteria become mutagenic and carcinogenic: Lessons from *H. pylori*. *Mutation Research/Genetic Toxicology*, 2010, 703 (1), pp.66-70. 10.1016/j.mrgentox.2010.07.014 . hal-01651959

**HAL Id: hal-01651959**

**<https://hal.science/hal-01651959v1>**

Submitted on 29 Nov 2017

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**When bacteria become mutagenic and carcinogenic: lessons from *H. pylori***

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*Keywords* : Infection, nucleotide modification, DNA repair, inflammation, cancer

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## **Abstract**

More and more convincing data link bacteria to the development of cancers. How bacteria act as mutagens by altering host genomes, what are the different strategies they develop and what consequences do they have on infection-associated pathogenesis are the main questions addressed in this review, which focuses in particular on *Helicobacter pylori* infection. *H. pylori* is a major risk factor for gastric cancer development. Its oncogenic role is mediated by the chronic active inflammation it elicits in the gastric mucosa, associated with its capacity to persistently colonize the human stomach. However, direct genotoxicity of *H. pylori* through the action of bacterial cytotoxin or resulting from a DNA damaging effect of its metabolic derivatives as nitroso compounds cannot be excluded. Numerous studies have investigated inflammation-associated DNA damaging activity and mutagenic response due to *H. pylori* infection in both human and animal models. Recent findings on its mutagenic effects at the nuclear and mitochondrial genome and related DNA damage are reviewed. This genotoxic activity associated with oxidative species produced during inflammation is linked to the decreased efficiency of DNA repair systems. DNA methylation, which plays an important role in the regulation of the host response to *H. pylori* infection, is also documented. Furthermore, *H. pylori* affects genome integrity by increasing activation-induced cytidine deaminase (AID), a DNA/RNA editing cytidine deaminase linking mutagenesis and tumorigenesis. These different strategies occurring during bacteria-host cell interaction, lead to nucleotide modifications and genome instabilities recognized as early events in the carcinogenesis process and contribute to the oncogenic properties of *H. pylori* infection.

## Introduction

Sixteen percent of cancer incidence throughout the world can be attributed to infectious agents. Although the viral origin of human cancers is well established, convincing data also link bacteria to cancer development [1]. *Helicobacter pylori* is associated with the majority of gastric cancers classified as intestinal-type adenocarcinoma and recognized as a type 1 carcinogenic agent [2] [3]. The associated gastric lesions are initiated by chronic superficial gastritis, atrophic gastritis, and intestinal metaplasia that evolve into dysplasia and adenocarcinoma [4] [5]. Chronic gastritis is the hallmark of *H. pylori* infection. Several *H. pylori* virulence factors such as variants of *vacA* encoding a vacuolating cytotoxin [6], and the presence of the *cag* pathogenicity island (Cag-PAI) are associated with the most severe gastric pathologies [7]. Recently, the CagA protein, a member of Cag-PAI has been shown to induce chromosomal instability by causing mitotic impairment [8]. However, *H. pylori* can also be considered as an indirect carcinogenic agent due to the chronic active inflammation it elicits in the gastric mucosa associated with its ability to escape immune response [9] and thus colonize the human stomach for decades. A combination of indirect and direct mechanisms make it likely that *H. pylori* contributes to gastric cancer development via complex interplay between chronic inflammation, bacterial effects on host cell physiology, probably changes in tissue stem cell homeostasis and the consequences of environmental triggering factors [10] [11]. The scope of this review is to report recent findings on the different strategies developed by *H. pylori* either directly or indirectly to induce DNA damage and host genome instability and the biological consequences of the latter on pathogenesis.

## **1. Mutagenic response induced by *H pylori* infection**

### ***1.a DNA damage and mutation at the nuclear genome***

Several reports have described a wide range of genetic instabilities in intestinal type gastric cancer and its precursor lesions, specially focusing on the tumor suppressor gene p53 [12] [13]. Most common mutational events are GC->AT transitions occurring at CpG dinucleotides and resulting from cytosine deamination. GC->AT transitions caused by carcinogenic N-nitrosamines from dietary amine and nitrates present in the acid gastric environment have been suggested [13]. Increased formation of nitrosamines has also been reported during acute and chronic inflammation. It can lead to deamination of 5-methylcytosine at CpG dinucleotides resulting in C to T transition [14]. GC->AT is the most common transition event found in european compared to AT->GC that is mainly found in asian population. More p53 mutations were found in *H. pylori* positive gastric cancer patients than in *H. pylori* negative ones [15]. CagA+ *H. pylori* strains are associated with severe gastric inflammation in the gastric mucosa. Gastric tumors from CagA+ *H. pylori* patients were found 3.7 times more likely to harbor p53 mutations than tumors from CagA- subjects [16]. Also in that case, mutations were predominantly insertions/deletions and GC->AT transitions that were found associated with precancerous gastric lesions in *H. pylori* positive patients [17]. In *K-ras*, GC->CG transversions occurred more frequently in *H. pylori*-associated chronic gastritis in patients with gastric cancer than those without it [18].

The development of gastric cancer lesions directly associated with the presence of *H. pylori* infection was demonstrated in mongolian gerbil model [19] [20]. Using the Big Blue mice assay [21] [22], we showed a direct gastric mutagenic effect induced by a chronic *H. pylori* infection [23]. A 5-fold increase in gastric mutation frequency after 6 months in comparison to non-infected mice was found with the presence of an active

gastritis. It is associated with high expression of the inducible nitric oxide synthase (iNOS) responsible for the production of NO that can react with superoxide to form peroxynitrite, leading to 8-nitroguanine (8-NG). The induced mutation spectra is mainly composed of transversions of GC to TA, AT to CG and AT to TA, suggesting the involvement of oxidative DNA damage [23]. Also, in *p53*<sup>+/-</sup> Big Blue mice chronically infected with *H. felis*, a cat isolate, an increase of mutations in the gastric mucosa associated with a higher risk of gastric neoplastic lesions has been confirmed [24]. During chronic inflammation, reactive oxygen and nitrogen species (ROS and RNS) are generated from inflammatory cells recruited in the gastric mucosa following *H. pylori* interaction with gastric epithelial cells, inducing mainly 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and 8-nitroguanine, known to elicit GC → TA transversions [25]. An immunohistochemistry analysis of gastric samples from patients with *H. pylori* positive gastritis and from patients in which it had been eradicated, correlated the increase of 8-oxodG and 8-NG in the framework of *H. pylori* infection [26]. Several teams presented data to claim that *H. pylori* infection is apparently the single most important factor in determining the levels of DNA oxidative damage as 8-oxodG in the gastric mucosa [27] [28,29]. The association between the presence of these DNA adducts and gastric mucosal atrophy and intestinal metaplasia was clearly established. Another pathway leading to oxidative DNA damage during *H. pylori* infection is the induction of polyamine oxidation resulting from spermine oxidase activity generating H<sub>2</sub>O<sub>2</sub> [30].

Like DNA, deoxyribonucleoside triphosphates (dNTP) pools are also targets for oxidative species. Mutations can also be generated by the incorporation of oxidized-dNTP into DNA by DNA polymerases [31], despite them being poor substrates [32]. Idaka et al reported the incorporation of 2-hydroxy-2'-deoxyadenosine 5'-triphosphate (2-OH-dATP) and 8-hydroxy-2'-deoxyguanosine 5'-triphosphate (8-OH-dGTP) by

human DNA polymerase  $\eta$  (hpol $\eta$ ), leading to G to T and A to C transversions respectively [33]. ROS and RNS produced by inflammatory cells can also induce base alkylation *via* lipid peroxidation (LPO). Etheno-modified ( $\epsilon$ -modified) bases are generated by the reaction of DNA with a major LPO product (*trans*-4-hydroxy-2-nonenal). Increased levels of etheno adducts are found in chronically inflamed human tissues and have been suggested as potential markers for assessing the progression of inflammatory cancer-prone diseases [34]. The role of etheno DNA adducts in the genotoxicity of *H. pylori* must be considered. As suggested by the multistep model of gastric carcinogenesis and despite the important role of inflammation due to oxidative and nitrative DNA damage, a direct genotoxic activity of *H. pylori* bacteria on the gastric mucosa cannot be excluded. *H. pylori* produces different substances able to promote DNA damage including cytotoxins and metabolic derivatives such as nitroso compounds. Moreover, ammonium derived from *H. pylori* urease activity, which allows bacteria to resist gastric acidity [35], can form mutagenic derivatives such as HNO<sub>2</sub> by oxidation. A direct genotoxic effect of bacteria is in agreement with *in vitro* studies on *H. pylori*-infected gastric epithelial cells which reported a release of free radicals, the induction of DNA damage and frameshifts and point mutations [36] [37].

***1b. H. pylori is also a mutagen for mitochondrial DNA***

MtDNA mutations have been detected in all types of tumors [38]. In gastric cancer, mtDNA instabilities occur at the early stage of tumorigenesis [39] [40]. They are more frequently found in *H. pylori*-positive patients with gastric cancer [41] [42]. An association between *H. pylori*-related peptic ulcers and mtDNA mutations has also been suggested [43]. In most of these studies, mtDNA mutations concerned deletion/insertion events in the mtDNA D-loop region. Recently, we have demonstrated the induction of mtDNA mutations in gastric epithelial cells chronically infected with *H. pylori* in both

the mitochondrial D-loop region and genes encoding subunits of the electron transport chain [44]. The mutation spectra consisted mainly in AT->GC and GC->AT transitions and single insertion. These data were also validated *in vivo* on gastric biopsies from *H. pylori*-infected patients with chronic gastritis [44]. We confirmed the induction of mtDNA mutation in the gastric mucosa of *H. pylori* infected mice after 12 and 18 months, including mainly AT->GC, AT->CG and single (G ;C) insertion [unpublished results]. By inducing mutation in the mitochondrial genome, *H. pylori* may impair oxidative phosphorylation metabolism, leading to an increase of ROS production and mtDNA damage. The close proximity of mtDNA to the respiratory chain and sources of reactive oxygen species may promote oxidative DNA damage. Oxidative stress has been recently demonstrated to lead to the degradation of mtDNA, as strand breaks and abasic sites prevail over mutagenic base lesions in ROS damaged mtDNA [45]. Another factor to be considered that can influence the mutation specificity at mtDNA is the reduced DNA repair availability in mitochondria that need to import DNA repair components.

In recent years, a major role of stem cells in gastric carcinogenesis has been proposed. Using mtDNA mutations as a marker of clonal expansion, McDonald *et al*, [46] reported that mutation originally occurs in the stem cell that colonizes a unit or crypt and spreads by fission until a mutated field of epithelial cells develops, subsequently leading via further mutational events to tumor development. A role of mtDNA mutation in the acquisition of metastatic properties of tumor cells has been demonstrated [47]. Using cytoplasmic hybrid technology, the endogenous mtDNA in a mouse tumor cell line with low metastatic properties was replaced by mtDNA from a highly metastatic cell line, mutated in the gene coding for NADH (reduced form of nicotinamide adenine dinucleotide) dehydrogenase subunit 6 (ND6). This mutation resulted in a deficiency of respiratory complex I activity and was associated with an overproduction of ROS. These



authors demonstrated that the presence of mutated mtDNA is associated with the acquisition of high metastatic ability for the receptor cells.

## **2. The *H. pylori*-induced mutator phenotype is associated with DNA repair impairment**

In 6 month *H. pylori*-infected mice, the expression of mismatch repair (MMR) components is down-regulated [44]. We also observed an induction of microsatellite instabilities (MSI) at gastric mucosa level. MSI is a hallmark of MMR deficiency frequently associated with colorectal and gastric adenocarcinoma [48] [49]. Deficiency in mismatch repair has also been confirmed in gastric biopsies from *H. pylori*-infected patients [50] [51]. During Base-Excision Repair (BER) abasic sites are repaired by apurinic/apyrimidinic (AP) endonuclease (APE1). The expression of APE1 was also observed to be downregulated in *H. pylori*-infected gastric epithelial cells (AGS) [44]. It should lead to the accumulation of abasic sites that can be converted by mutation during DNA replication or DNA repair. DNA damage induced by ROS and RNS are recognized by alkyladenine DNA glycosylase (Aag) involved in BER. Recently, Meira *et al* reported that a deficiency in BER alkyladenine DNA glycosylase (Aag) in mice triggers the development of a preneoplastic stage [52]. The expression of O6-Methylguanine DNA methyltransferase (MGMT) involved in the repair of O6-Methylguanine (O6-mG) preventing GC to AT transitions is also decreased by *H. pylori* infection [53]. This is due to CpG methylation at the *MGMT* gene promoter region and associated with chronic active gastritis. Thus the induction of genome instability and mutations during *H. pylori* infection is directly related to a decrease of the DNA repair efficiency of host cells, thereby influencing the associated mutation spectra and promoting malignant transformation.

### 3. DNA methylation and relation with inflammation

Epigenetic alterations have been suggested as key initiating events in tumorigenesis occurring very early in cancer development [54]. As recently reviewed, the cancer epigenome is marked by genome-wide hypomethylation and site specific CpG island promoter hypermethylation [55]. The resulting 5-methylcytosine is relatively unstable and prone to spontaneous deamination changing it to thymine, a source of endogenous mutagenesis. In several studies, aberrant DNA methylation in gastric biopsies from *H. pylori*-positive patients has been correlated with higher risks of gastric cancer [56] [57] [58] [59]. The role of *H. pylori* in the induction of DNA hypermethylation is confirmed by a low incidence of DNA methylation in patients after eradication of the infection [60] [61] [62]. DNA hypermethylation contributes to tumorigenesis by silencing tumor suppressor genes. It can also indirectly modulate gene expression, by downregulating transcription factors and DNA repair gene expression [63]. We have recently demonstrated that *H. pylori* downregulates the gene expression of upstream stimulatory factors USF1 and USF2 by DNA hypermethylation of their promoter region [64]. USF1 and USF2 are pleiotropic transcription factors that regulate the expression of genes related to immune response, cell proliferation and chromosome stability. Thus, by modulating the levels of such pleiotropic regulators, DNA methylation plays an important role in host response to *H. pylori* infection. As mentioned above, *H. pylori* promotes genetic instability through the decrease of DNA repair gene expression. In agreement with this, CpG hypermethylation has been described in the presence of infection in the promoter region of *MLH1*, a component of MMR and in the promoter region of *MGMT* involved in BER DNA repair [65] [53].

Aberrant DNA hypermethylation is frequently associated with chronic inflammation, as observed in non-cancerous tissues of patients with inflammation-

associated cancers. In the case of gastric cancer, a link between polymorphonuclear infiltration and hypermethylation of CpG islands has been reported by Kaise *et al* [66]. Using the Mongolian gerbil model, Niwa *et al* demonstrated that *H. pylori*-associated DNA methylation is composed of transient components that disappeared after eradication of the infection and permanent components, mainly associated with chronic inflammation [67]. These data were also confirmed in humans [68]. In line with this, we showed that DNA hypermethylation induced by *H. pylori* in the gastric mucosa of mice chronically infected for 18 months occurred in the context of dysplastic lesions [64]. Recently, Sepulveda *et al* suggested that permanent DNA methylation after *H. pylori* eradication should occur in stem cells, thus promoting tumorigenesis [53]. How inflammation triggers DNA methylation has not been completely elucidated. Several years ago, Henderson *et al* reported the presence of HOCl from activated neutrophils and HOBr from activated eosinophils in the area of tissue inflammation [69]. HOCl and HOBr are known to react with DNA to form, among other products, 5-chlorocytosine and 5-bromocytosine, which are mutagenic [70]. Valinluck *et al* have established a potential role for these halogenated cytosine damage products in methylation changes [71]. Halocytosines were revealed to be as efficient as 5-methylcytosine in enhancing the binding of the methyl-CpG-binding protein 2 in directing DNA methylation at CpG. These studies provide further insights into inflammation-related tumorigenesis mechanisms and DNA methylation [72], that must be investigated in the context of *H. pylori* infection.

#### **4. Activation-induced cytidine deaminase (AID), a link between inflammation and tumorigenesis**

Activation-induced cytidine deaminase (AID) is a DNA/RNA editing cytidine deaminase which converts cytosine into uracil. It plays an essential role in the diversity of immunoglobulins in activated B cells, by somatic hypermutation (SHM) and class switch

recombination (CSR) [73]. AID is mutagenic due to its cytidine deaminase activity, leading GC->AT transitions. Its mutagenic properties have been recently analysed in non-B cells, using a system based on the reversion of a nonsense mutation in an EGFP gene [74]. Under these conditions, transiently transfected DNA can be mutated in an AID-dependent manner with the induction of mutation both at G:C and A:T. The constitutive expression of AID in transgenic mice is correlated with tumor development in various organs associated with high mutation frequencies [75]. The role of AID in tumorigenesis *via* its mutagenic activity has been recently demonstrated in inflammation related-human cholangiocarcinoma [76]. In this study, a pro-inflammatory cytokines-dependent aberrant expression of AID in biliary cells resulted in somatic mutations in tumor related-genes such as *p53*, *c-myc* and the promoter region of *INK4A/p16* sequences. This induction of AID depends on the activation of the proinflammatory transcription nuclear factor  $\kappa$ B (NF- $\kappa$ B). An upregulation of AID with an accumulation of nucleotide alterations in the *p53* tumor suppressor gene in *H. pylori*-infected gastric cells has been reported [77]. In this case, AID upregulation is dependent on the NF- $\kappa$ B activation pathway, known to play an important role in inflammation-associated carcinogenesis [78] [79]. High levels of AID were also observed in *H. pylori* positive gastritis and gastric cancer tissues from human biopsies [77]. Recent data from our group confirmed the direct effect of *H. pylori* infection on the induction of AID expression associated with active chronic gastritis in mice [unpublished results]. These results illustrate the ability of *H. pylori* to affect genome integrity indirectly by inducing a DNA mutator enzyme usually active in normal B-cells.

## **Conclusion**

In conclusion, the recent findings reported in this review confirm the capacity of *H. pylori* infection to induce genetic instabilities and DNA repair deficiency in host cells. Its

mutagenic effect can also result from the induction of high levels of a DNA mutator like AID. These genotoxic properties are directly linked with the chronicity of the infection. Current knowledge and in vitro data lead to the assumption that the oncogenic properties of *H. pylori* infection result from two mechanisms: i) a direct *H. pylori* genotoxic effect resulting from the action of its virulent factors and products stemming from bacterial metabolism ; ii) the major role played by gastric inflammation, mainly attributed to ROS and RNS-mediated DNA and cell damage, that is well documented. Thus, *H. pylori* related gastric cancer lesions are the results of this complex interplay. *H. pylori* is also a source of epigenetic alterations, and up to now DNA methylation is the best characterized associated epigenetic modification. However the study of other epigenetic events such as histone modification and the involvement of microRNAs are promising fields for a better understanding of the relation between *H. pylori* infection and cancer. Other bacteria are suspected to be associated with tumor development such as *Salmonella typhi* and gallbladder cancer, *Chlamydomphila pneumoniae* and lung cancer, *Streptococcus bovis* and *Escherichia coli* in colorectal cancer [1]. Better knowledge of the mechanisms involved in the relation between *H. pylori* and gastric cancer will lead to further insights in the genotoxicity associated with these different bacterial pathogens and their consequences on the development of malignancies.

**Conflict of interest**

There is no conflicting interest.

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