

Review

Molecular Mechanism of Gastric Carcinogenesis in *Helicobacter pylori*-Infected Rodent Models

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Abstract: Since the discovery of *Helicobacter pylori* (*H. pylori*), many efforts have been made to establish animal models for the investigation of the pathological features and molecular mechanisms of gastric carcinogenesis. Among the animal models, Mongolian gerbils and mice are particularly useful for the analysis of *H. pylori*-associated inflammatory reactions and gastric cancer development. Inhibitors of oxidative stress, cyclooxygenase-2 (COX-2) and nuclear factor- κ B, exert preventive effects on chronic gastritis and the development of adenocarcinomas in *H. pylori*-infected gerbils. Genetically-modified mouse models, including transgenic and knockout mice, have also revealed the importance of p53, COX-2/prostaglandin, Wnt/ β -catenin, proinflammatory cytokines, gastrin and type III mucin in the molecular mechanisms of gastric carcinogenesis. Microarray technology is available for comprehensive gene analysis in the gastric mucosa of mouse models, and epigenetics, such as DNA methylation, could be an alternative approach to correlate the observations in animal models with the etiology in humans.

Keywords: gastric cancer; *Helicobacter pylori*; molecular mechanism; carcinogenesis; mouse; Mongolian gerbil

1. Introduction

Gastric cancer is the fourth most common cancer and second leading cause of cancer-related death in the world [1]. Since *Helicobacter pylori* (*H. pylori*) was discovered about 30 years ago [2,3], a lot of epidemiological and experimental studies have revealed a significant relationship between *H. pylori* infection and chronic/atrophic gastritis, peptic ulcer, intestinal metaplasia, gastric lymphoma or cancer development [4–14]. In 2001, Uemura *et al.* confirmed that stomach cancers develop only in *H. pylori*-infected patients, but none of the uninfected group [15]. Based on the epidemiological findings, *H. pylori* was defined as a “definite carcinogen” by the World Health Organization/International Agency for Research on Cancer (WHO/IARC) in 1994 [16]. Since then, many experimental studies have been performed to investigate the mechanisms of gastric carcinogenesis using animal models, as well as clinical samples. Among animal models, Mongolian gerbils and mice are particularly useful for the analysis of *H. pylori*-associated gastric disorders [17,18]. In this article, we will review the research to date for the analysis of the molecular mechanisms of *H. pylori*-associated gastric carcinogenesis using rodent models.

2. Establishments of Rodent Carcinogenesis Models

2.1. Establishment of Chemical Carcinogenesis Model

Several rodent models have been established to investigate the pathological features and molecular mechanisms of stomach carcinogenesis. These include rats, mice and Mongolian gerbils, and chemical carcinogens were used to induce gastric cancer. Some chemicals used in the early studies, such as benzo[a]pyrene [19], 3-methylcholanthrene [20] and 2-acetylaminofluorene [21], showed a low incidence of gastric lesions in rats. However, it has been reported that oral administration of 4-nitroquinoline 1-oxide (4-NQO) and 4-hydroxyaminoquinoline 1-oxide (4-HAQO) could induce adenocarcinomas of glandular stomach, as well as various other tissues in rats and mice [22,23]. In addition, Sugimura and Fujimura established a rat model using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) in drinking water to induce gastric adenocarcinomas with relatively high incidence [24]. Similarly, *N*-methyl-*N*-nitrosourea (MNU) was shown to induce adenocarcinomas in the glandular stomach of mice, including BALB/c [25], C3H [26] and other strains [27]. In the MNU-treated mice, both differentiated and undifferentiated adenocarcinomas typically develop, showing more significant cellular atypia compared with the MNNG-treated rats. In 1998, Tatematsu *et al.* have demonstrated that stomach cancers, including well- and poorly-differentiated adenocarcinoma and signet-ring cell carcinoma, also develop in Mongolian gerbils (*Meriones unguiculatus*), both by MNU or MNNG treatment [28]. The establishment of mouse models has provided new approaches to clarify the molecular mechanisms of gastric carcinogenesis by using genetically-modified animals.

2.2. Establishment of *H. pylori* Infection Models

Since the discovery of *H. pylori*, various types of experimental animals have been attempted to develop *H. pylori* infection models, but none of the early models could sufficiently reproduce the human situation [29,30]. In 1990, *Helicobacter felis* (*H. felis*), a novel *H. pylori*-related bacterium isolated from cat stomach, was found to be able to colonize the glandular stomach and induce acute and chronic gastritis in germ-free mice [31]. After that, *H. pylori* isolated from human clinical samples were also shown to cause chronic active gastritis in mouse models [32–34]. The Sydney strain of *H. pylori* (SS1) was established by screening of clinical isolates and showed high colonizing ability in C57BL/6 and BALB/c mice [35]. In addition, a Mongolian gerbil model was successfully established and shown to induce *H. pylori*-associated gastric disorders, including chronic active gastritis, peptic ulcers and intestinal metaplasia, which are closely similar to human [36].

H. pylori infection increases the incidence of MNU- and MNNG-induced adenocarcinomas in gerbils [37–39]. Although early reports suggested that *H. pylori* infection alone can induce gastric adenocarcinomas in gerbils [40–42], subsequent studies have demonstrated by detailed histopathological assessment that gastric carcinomas are rarely observed in animals treated only with *H. pylori* infection [28,37–39,43], suggesting that *H. pylori* is a strong promoter of gastric carcinogenesis, rather than an initiator. The *H. pylori*-infected and carcinogen-treated gerbil model has been widely used for the investigation of the underlying mechanisms of gastric cancer development [17,18].

While a number of new insights have been provided from the *Helicobacter*-infected rodent models, there are limitations that should be considered. Most rodent models take months to a year to develop the gastric lesion expected, and almost all gastric cancers in these models are not metastatic. Cytotoxin-associated gene A (CagA), one of the bacterial virulence factors located in cag pathogenicity islands (cagPAI) of the bacterial genome, is known to be associated with the risk of human stomach cancers [44]. *H. felis* lacks cagPAI and VacA, a vacuolating toxin [45]. Although both two major strains of *H. pylori*, ATCC43504 (also known as NCTC11637) and SS1, possess CagA, the SS1 may not express functional CagA protein [46,47]. Other considerations include the dependency on the gender, diet and genetic background of mice [48]. Taken together, it is considered that CagA-containing strains of *H. pylori* play an especially important role in the investigation of gastric carcinogenesis.

3. Mechanisms of Gastric Carcinogenesis *in Vivo*

Three general factors are considered to impact *H. pylori*-related gastric carcinogenesis: bacterial virulence factors, including CagA, the genetic susceptibility of the host and the environment [49]. As the environmental factor, diet has particularly attracted attention as a major determinant, and epidemiological and experimental studies have demonstrated that consumption of certain natural products can lower gastrointestinal cancer risk in humans and animal models through activation of antioxidative activity and inhibition of inflammatory pathways, such as COX-2, NF- κ B and other proinflammatory cytokines. Among the host genetic factors, gene polymorphisms of proinflammatory cytokines are shown to be associated with the susceptibility to gastric carcinogenesis. Gene manipulation technology enabled us to examine the roles of the specific gene in the mechanisms of *H. pylori*-induced chronic gastritis and cancer development.

3.1. Use of Inhibitors

3.1.1. Antioxidants

It has been suggested that inflammation-associated oxidative stress exerts significant effects on gastric carcinogenesis through upregulation of DNA damage [50]. Inhibition of *H. pylori*-induced gastritis and oxidative stress is considered as one of the promising approaches to prevent gastric cancer, because the major determinant factor of stomach carcinogenesis is the degree of *H. pylori*-induced inflammation [51]. To address this hypothesis, Cao *et al.* examined the inhibitory effect of 4-vinyl-2,6-dimethoxyphenol (canolol), an antioxidant obtained from crude canola oil, on chronic gastritis and gastric cancer development in *H. pylori*-infected gerbils [52]. As a result, *H. pylori*-induced gastritis, the expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) and serum 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels were significantly decreased in canolol-treated gerbils compared with the non-treated groups. In addition, the incidence of gastric adenocarcinomas was also reduced by canolol treatment, indicating that oxygen radical scavengers could prevent *H. pylori*-associated gastritis and carcinogenesis in gerbils. Interestingly, the canolol treatment did not affect the viable *H. pylori* count, suggesting that the degree of *H. pylori*-induced inflammation is a more important determining factor than the existence of the bacteria. Other naturally-derived compounds, such as sulforaphane from broccoli sprouts [53,54], and nordihydroguaiaretic acid found in the creosote bush [55], a common shrub of North America, have been also shown to be effective for the prevention of *H. pylori*-induced gastric disorders in rodent models through the antioxidative activity.

3.1.2. COX-2 Inhibitors

COX-2 has been shown to be involved in the processes of inflammation and carcinogenesis [56], and previous studies indicate that COX-2 expression is associated with *H. pylori*-induced gastritis and stomach carcinogenesis in humans [57,58]. *H. pylori* infection and excessive salt intake synergistically enhance COX-2 and iNOS expression in the gastric mucosa of gerbils [59]. Thus, the inhibition of COX-2 may be a promising target for reducing the risk of gastric cancer. Actually, COX-2 selective inhibitors, such as etodolac and celecoxib, have been shown to exert preventive effects on the stomach cancer of *H. pylori*-infected gerbils [60,61].

Recent epidemiological studies have demonstrated that eradication of *H. pylori* has preventive effects on gastric cancer development [62,63]. However, since not all tumors are prevented by *H. pylori* eradication [64], the search for new approaches and alternative therapies for the prevention of stomach carcinogenesis after eradication continues to be very important. Because inflammatory reactions against the bacterium disappear after eradication, it is necessary to target factors directly regulating the proliferation and progression of tumor or precancerous cells for the prevention of gastric carcinogenesis after eradication. It has been demonstrated that COX-2 inhibitors could regress the early stage tumors in the intestine of mice and rats [65,66]. Therefore, there is another possibility that COX-2 inhibitors could be applicable to the regression of the remaining precancerous lesion and prevention of gastric cancer occurrence after *H. pylori* eradication.

3.1.3. NF- κ B inhibitors

Nuclear factor- κ B (NF- κ B) plays a central role in host inflammatory responses and carcinogenesis [67]. *H. pylori* infection activates the NF- κ B signaling pathway, and NF- κ B-mediated cytokine expression is essentially involved with *H. pylori*-induced gastritis in gerbils and mice [68–71]. Ogura *et al.* reported that the degree of gastritis induced by a mutant strain of *H. pylori* lacking capacity for NF- κ B activation is lower than that with wild-type infection in gerbils [72]. Therefore, it is considered that inhibition of NF- κ B could be a target for the prevention of *H. pylori*-associated gastric cancer [73]. Caffeic acid phenethyl ester (CAPE), a naturally-derived NF- κ B inhibitor, was analyzed on *H. pylori*-induced chronic gastritis using the gerbil model [74]. CAPE treatment significantly attenuated infiltration of neutrophils and mononuclear cells and the expression of the NF- κ B p50 subunit and phospho-I κ B- α in the antrum of *H. pylori*-infected gerbils. The proliferative activity of epithelial cells, both in the antrum and corpus, were markedly reduced by CAPE treatment. In addition, mRNA expression of inflammatory factors, such as tumor necrosis factor- α (*Tnf- α*), interferon- γ , interleukin (*Il*)-2, *Il*-6, KC (*Il*-8 homologue) and *iNos*, was significantly decreased in the pyloric mucosa. These results indicate that CAPE has inhibitory effects on *H. pylori*-induced gastritis in gerbils through the suppression of NF- κ B activation and may have the potential for the prevention and therapy of *H. pylori*-associated gastric disorders [74].

3.1.4. Statins

Statins, potent inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, are widely used drugs for the treatment of hypercholesterolemia, with beneficial effects on cardiovascular disease [75,76]. Several studies have suggested that statins have chemopreventive effects on various types of cancers [77–79]. To clarify the effects of statins on gastric carcinogenesis, pitavastatin was examined in *H. pylori*-infected gerbil and mouse models [80]. The incidences of *H. pylori*-associated gastric adenocarcinomas and the degrees of chronic gastritis were not decreased by pitavastatin with upregulation of *Il*-1 β and *Tnf- α* mRNA expression in the pyloric mucosa. Interestingly, serum total cholesterol (T-Chol), triglyceride (TG) and low-density lipoprotein (LDL) levels were significantly increased by pitavastatin treatment in the *H. pylori*-infected groups. In the short-term study, *H. pylori*-infected gerbils and mice also showed upregulation of TG levels by pitavastatin, whereas T-Chol was markedly reduced, and LDL exhibited a tendency for decrease in non-infected animals. These findings indicate that pitavastatin has no suppressive effects on chronic gastritis and stomach carcinogenesis in *H. pylori*-infected rodent models, and the *H. pylori* infection and following severe chronic gastritis interfere with the cholesterol-lowering effects of pitavastatin [80].

3.2. Use of Genetically-Modified Animals

Genetically-modified rodent models have been used for the investigation of the mechanisms of gastric carcinogenesis. The target genes include the oncogenes and tumor-suppressor genes directly associated with the transformation of gastric epithelial cells, the signaling pathways and cytokines involved in the proinflammatory responses induced by *Helicobacter* infection; and the environmental regulatory factors for colonization and proliferation of *H. pylori* in the glandular stomach.

3.2.1. p53 Tumor Suppressor Gene

The p53 tumor suppressor gene is known to be frequently mutated in various types of human cancer [81]. However, MNNG-induced rat stomach adenocarcinomas had a mutation of the p53 gene in only one of 10 cases [82]. In addition, no mutations in exons 5–8 were found in a total of 30 gastric tumors in MNU-treated mice [83]. In 1992, the p53 knockout mouse has been established by Donehower *et al.* as a powerful tool for functional analysis of carcinogenesis [84–86]. Yamamoto *et al.* have investigated the susceptibility of p53 nullizygote (–/–), heterozygote (+/–) and wild-type (+/+) mice to MNU-induced pepsinogen altered pyloric glands (PAPGs) and stomach carcinogenesis [87]. PAPGs are putative precancerous lesions immunohistochemically stained weakly or negative for pepsinogen 1 (Pg 1) in mice and rats [88–90]. At five weeks, there were more PAPGs both in slightly irregular glands and the normal-looking mucosa of MNU-treated p53 (–/–) mice than the control mice with consistent expression of Pg 1 in the pyloric gland. The frequency of PAPGs in MNU-treated p53 (–/–) mice was significantly elevated compared with the values for MNU-treated p53 (+/+) and (+/–) mice and the control groups. At 15 weeks, adenomas were observed in two of 21 p53 (+/–) and six of 10 p53 (–/–) mice, and adenocarcinoma was detected in one of 10 p53 (–/–) animals. There was a significant tendency toward malignancy in the tumors developed in p53 (–/–) mice compared with (+/–) and wild-type mice. At 40 weeks, no significant difference in the incidences of gastric adenoma and adenocarcinoma between p53 (+/+) and (+/–) mice was observed [87,91]. PCR-single strand conformation polymorphism (SSCP) analysis revealed that none of the 68 gastric tumors derived from the 15- and 40-week experiments has any mutations in the p53 gene, although the simultaneous development of lymphoma and sarcoma in MNU-treated groups showed mutations in the wild-type allele of p53 [87]. These results indicate that p53 (–/–) mice are more susceptible to MNU-induced gastric carcinogenesis than p53 (+/–) and wild-type mice. Thus, it is suggested that p53 may act as a gatekeeper in the gastric carcinogenesis of rodents, rather than as a direct target of chemical carcinogen.

3.2.2. COX-2/Prostaglandin

COX-2 is a rate-limiting enzyme for prostanoid biosynthesis, and prostaglandin E2 (PGE2) is known to be most important for cancer development. In 2004, Oshima *et al.* established K19-C2mE mice simultaneously expressing COX-2 and microsomal prostaglandin E synthase (mPGES)-1 under a keratin 19 (K19) promoter in the gastric epithelial cells, and the transgenic mice developed proliferative lesions in the glandular stomach with significant infiltration of macrophages [92]. They revealed the importance of the COX-2/mPGES-1 pathway in *Helicobacter*-associated gastric carcinogenesis, since *H. felis* infection also upregulated epithelial expression of COX-2 and PGE2. TNF- α -dependent inflammation is required for the development of proliferative lesions in the COX-2/mPGES-1 mice [93]. COX-2 and mPGES-1 expression progressed metaplastic lesions observed in K19-Wnt1 transgenic mice to dysplastic gastric tumors [94]. In addition, additional expression of noggin, a bone morphogenetic protein (BMP) antagonist, in K19-C2mE mice caused gastric hamartoma that resembles human juvenile polyposis syndrome [95]. CD44-positive stem-like cells are candidates for the origin of gastric tumor in K19-Wnt1/C2mE mice and triggered by PGE2-induced inflammation and Wnt signaling [96]. The germ-free colony of K19-Wnt1/C2mE mice

develops less gastric tumors, and additional *H. felis* infection recovers the tumorigenesis, suggesting that bacterial infection and inflammation play a critical role in gastric carcinogenesis [97]. Takasu *et al.* has demonstrated that *H. pylori* (SS1) infection and MNU treatment could induce gastric adenocarcinomas, not only in the antrum, but also in the corpus of K19-C2mE mice, providing a better model for increasing proximal gastric cancers [98].

3.2.3. Wnt/ β -Catenin

The activation of Wnt/ β -catenin signaling mainly caused by mutations in exon 3 of the β -catenin gene is found in 30%–50% of gastric cancers, suggesting an important role of Wnt signaling in stomach carcinogenesis [99]. In MNNG-induced rat adenocarcinomas, four of twenty-two tumors showed nuclear localization of β -catenin with the characteristic mutation of exon 3 [100]. This study suggested that β -catenin mutations could be associated with the progression of chemical-induced rat adenocarcinoma at the late stage. In the gerbil model, only one of forty-five adenocarcinomas induced by *H. pylori* infection and MNU treatment showed nuclear accumulation of β -catenin with gene mutation [101]. Nuclear localization of β -catenin in stomach cancers is more frequently observed in mouse models. It has been reported that *H. pylori* infection enhances the activation of β -catenin in the gastric carcinogenesis of the pyloric region, especially in K19-C2mE transgenic mice [98]. Du *et al.* suggested that Sox17 expression prevents malignant progression of gastric tumors in K19-Wnt1/C2mE mice through the regulation of Wnt activity [102].

3.2.4. Proinflammatory Cytokines

H. pylori infection-mediated cytokine expression is essentially involved in the development of chronic gastritis and stomach tumors. TNF- α and IL-1 β are considered to be particularly important, because epidemiological studies have suggested that their polymorphisms are associated with the increased risk of gastric carcinogenesis [103–105]. Oshima *et al.* recently reported that additional knockout of TNF- α or TNF- α receptor results in the significant suppression of gastric tumor development in the K19-Wnt1/C2mE mice [106]. *H. pylori*-associated gastric carcinogenesis was also attenuated in IL-1 β knockout mice with decreased infiltration of neutrophils and macrophages [107]. MyD88 is one of the adaptor molecules in host inflammatory responses, and a recent study demonstrated that MyD88-deficient mice show the early onset of *Helicobacter*-induced gastric dysplasia with increased expression of TNF- α , IL-1 β , INF- γ and IL-6 [108]. Thus, these studies indicate that proinflammatory cytokines and related pathways could be effective targets of preventive and therapeutic strategies for stomach cancer.

3.2.5. Gastrin

The association between hypergastrinemia and gastric carcinogenesis remains unclear. Most patients infected with *H. pylori* exhibit relatively low serum gastrin levels along with the progression of atrophic gastritis. Actually, it has been reported that gastrin-deficient mice show increased gastric inflammation and tumor development mainly in the antrum [109,110]. On the other hand, Wang and their colleagues have demonstrated that chronic hypergastrinemia in the insulin-gastrin (INS-GAS)

transgenic mice could induce gastric cancer in the corpus, and *H. felis* and *H. pylori* infection synergistically enhances the progression of tumors [111,112], suggesting that gastrin exerts distinct functions in the antrum and corpus. The INS-GAS mice have been widely used for various fields of investigation of gastric carcinogenesis [113].

3.2.6. Type III Mucin

The gastric mucosa are covered with two types of mucin, surface and gland mucins. Gland mucin is secreted from mucous neck cells and pyloric gland cells and contains *O*-linked oligosaccharides (*O*-glycans) with terminal α 1,4-linked *N*-acetylglucosamine residues (α GlcNAc) [114]. It has been revealed that *H. pylori* could colonize and proliferate not in the gland mucous layer, but in the surface mucous layer, because the *O*-glycans have antimicrobial effects on *H. pylori* [115,116]. Karasawa *et al.* established A4gnt(−/−) mice, completely lacking α GlcNAc expression in the gastric gland mucin, and showed the spontaneous development of gastric adenocarcinomas without *H. pylori* infection [117]. The expression of pro-inflammatory cytokines and growth factors is increased in the gastric mucosa of A4gnt(−/−) mice, suggesting that the absence of α GlcNAc triggered inflammatory responses followed by gastric tumorigenesis. In human cases, the loss of α GlcNAc in well-differentiated adenocarcinoma with MUC6 expression is shown to be significantly associated with tumor malignancy and poor prognosis [118].

3.3. Microarray and Epigenetics

3.3.1. Microarray

Microarray technology has been applied to investigate global gene expression patterns in both human samples and animal models of gastric disorder [119–123]. While there are many reports investigating the gene expression profiles of *H. pylori*-treated gastric cell lines, cell culture studies do not always reflect the *in vivo* microenvironment, including host immune responses and stromal-epithelial interactions in cancers. Since there is little information available for the gerbil genome, attention has focused on mouse models. Itadani *et al.* demonstrated that gastric tumors in K19-Wnt1/C2mE transgenic mice are closely similar to intestinal-type stomach cancer regarding the gene expression profiles [124]. Among thirty-five candidate genes upregulated in *H. pylori*-infected and high-salt diet-treated mice, *Cd177* expression is found to be a novel prognostic factor of patients with advanced gastric adenocarcinoma [125]. Thus, it is considered that the mouse model is suitable for the investigation of the gene expression profile associated with stomach carcinogenesis.

3.3.2. Epigenetics

DNA methylation is one of the epigenetic mechanisms for gene regulation and deeply associated with cancer development of various tissues. Aberrant DNA methylation is induced by aging and chronic inflammation, including ulcerative colitis, chronic hepatitis and *H. pylori*-induced gastritis [126]. Niwa *et al.* revealed that *H. pylori*-associated inflammation, rather than infection itself, is responsible for inducing the aberrant DNA methylation in gastric epithelial cells using the gerbil model [127]. The authors also demonstrated that specific types of inflammation caused by *Helicobacter* infection, not

the increased cell proliferation, has a strong potential to induce aberrant DNA methylation in the gastric mucosa [46]. A DNA demethylating agent, 5-aza-2'-deoxycytidine (5-aza-dC), could prevent gastric carcinogenesis in *H. pylori*-infected gerbils, suggesting that the suppression of aberrant DNA methylation is a target for the prevention of stomach cancers [128]. In addition, novel risk markers of gastric cancer were identified by the microarray analysis for hypermethylated CpG islands [129]. A recent study revealed that CagA of *H. pylori* causes aberrant epigenetic silencing of let-7 followed by upregulation of Ras oncoprotein using *in vitro* experiments and an *in vivo* mouse model [130].

4. Conclusions

H. pylori infection is one of the most important risk factors for gastric carcinogenesis in human stomach. Since the discovery of *H. pylori*, the Mongolian gerbil and mouse have become useful model animals for the investigation of stomach carcinogenesis, the search for chemopreventive agents and molecular mechanisms. As already revealed by the gerbil model, *H. pylori* itself is a potent promoter in stomach carcinogenesis through chronic gastritis. The genetically-modified mouse models play a significant role to clarify what kind of molecules or genes act as a bridge between the *H. pylori*-induced inflammatory process and gastric cancer development. The global gene expression analysis and epigenetic approaches will become much more important for solving the complicated interaction.

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Author Contributions

T. Toyoda, M. Yamamoto, S. Takasu, K. Ogawa, M. Tatematsu and T. Tsukamoto all participated in designing, writing and editing of the review.

Conflicts of Interest

The authors declare no conflict of interest.

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