G-tail telomere HPA: simple measurement of human single-stranded telomeric overhangs

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Accurate measurement of telomeric 3′-overhang (G-tail) lengths is essential for investigation of the biological effects of telomere dysfunction. G-tail telomere hybridization protection assay (Gt-telomere HPA) has the advantages of being simple to perform, accurate and highly sensitive for G tails as short as 20 nucleotides. Furthermore, Gt-telomere HPA is specific and quantitative for human G tails, and can be used to assay cell lysates as well as genomic DNA.

Telomeric DNA is composed of many 5′-TTAGGG-3′ repeats. The terminus of each telomeric DNA has a single-stranded, 3′ overhang of between 75 and 300 bases in the G-rich strand, the so-called G tail[1,2]. Telomeric G tails are essential for proper telomere function[3,4].

Several methods are available for measuring the lengths of telomeric G tails (**Table 1**)[5–7]. Although the telomeric-oligonucleotide ligation assay (T-OLA)[6], the primer extension–nick translation (PENT) and the 3′ overhang protection assay[5] are all suitable for measuring G tails, they are complicated assays that require at least two days to complete. A further drawback is that they are difficult to apply in large-scale and high-throughput screening of samples. To overcome these problems, we have developed a new method for measuring telomeric G tails using a hybridization protection assay (Gt-telomere HPA). HPA has been used previously for detection of total telomere length and telomerase activity[8,9]. Our new method is based on an HPA format that uses oligonucleotide probes labeled with a highly chemiluminescent acridinium ester. Gt-telomere HPA is a single tube–based assay that can be completed in less than 40 min (**Table 1** and **Fig. 1a**). Whereas Gt-telomere HPA cannot be used to measure either variation in G-tail sizes within cells or the lengths of the G tails of individual chromosomes, the method has several key advantages: sensitivity for detection of short G tails, direct applicability to cell lysates, and the potential for large-scale screening using a 96-well format luminometer.

To determine the sensitivity and specificity of Gt-telomere HPA, we incubated serial dilutions of 84-mer telomere oligonucleotides, 5′-$(TTAGGG)_{14}$-3′, with 3×10^7 relative light units of luminescence (rlu) of 29-mer telomere HPA probes (**Supplementary Methods** online). We observed a linear increase in signal intensity with increasing oligonucleotide amount across the range 0.05 fmoles to 10 fmoles (**Fig. 1b**). The HPA probes can therefore be used to detect target mammalian telomere DNA sequences (**Supplementary Fig. 1** online). In a similar experiment, we obtained a linear response over a range of 1 μg to 20 μg of nondenatured genomic DNA (**Fig. 1c**). Thus, we now typically use 5 μg of nondenatured genomic DNA in this assay. In parallel experiments, we treated DNA samples with exonuclease I (ExoI) to remove single-stranded nucleotides in the 3′ to 5′ direction, thus deleting the telomere G tails. All the samples were ExoI-sensitive (**Fig. 1c**), confirming that the luminescent signals detected were specifically telomeric, single-stranded G tails. In contrast, treatment with T7 exonuclease, which removes the telomeric C strand in a 5′ to 3′ direction, thereby increasing 3′ telomeric G tails, increased the signals in a time-dependent manner (**Supplementary Fig. 2** online). At 90 s, the observed rlu value indicated that G tails with a mean length of approximately 1,600 nucleotides (nt) had been generated (**Supplementary Fig. 3** online). Taken together, the results of these enzyme-treatment experiments showed that Gt-telomere HPA can be used to specifically and quantitatively detect telomeric G tails.

Table 1 | Comparison of Gt-telomere HPA with known techniques to determine G-tail length

Method	Detection range (nt)	Detection time	Radioisotope	Assay in crude extracts	Electrophoresis	Determination of G-tail size distribution	High-throughput analysis
T-OLA	24–650	2 d	Required	Not possible	Required	Possible	Not possible
PENT	130–210	2 d	Required	Not possible	Required	Possible	Not possible
Electron microscopy	225–650	2 d	Required	Not possible	Required	Possible	Not possible
3′ overhang protection assay	45–384	2 d	Required	Not possible	Required	Not possible	Not possible
Gt-telomere HPA	20–1,600	40 min	Not required	Possible	Not required	Not possible	Possible[a]

[a]96-well plate screening is available.

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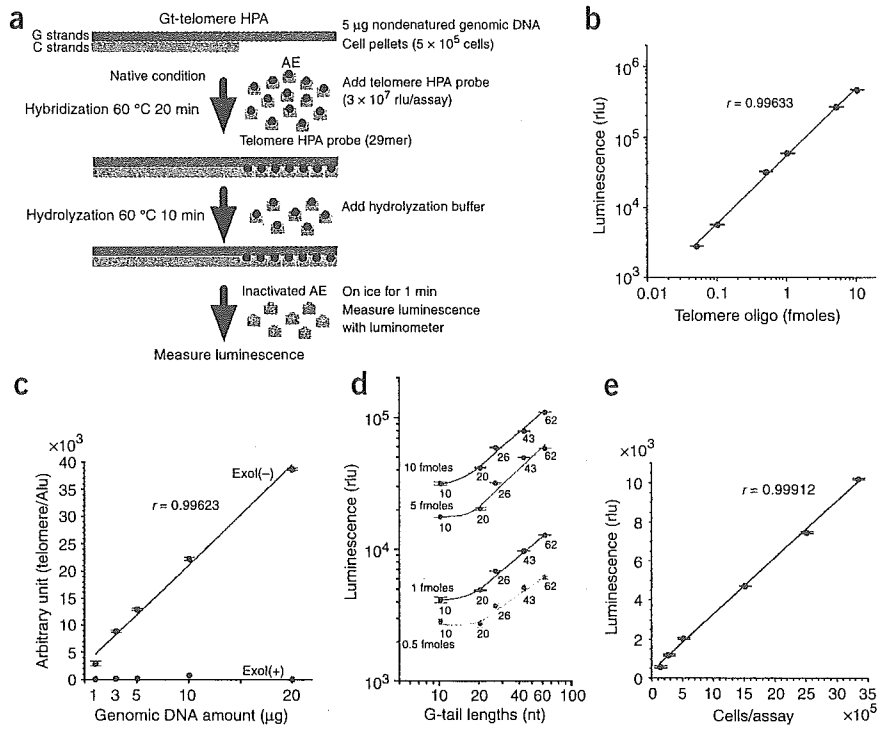

Figure 1 | Strategy, linearity, specificity and sensitivity of Gt-telomere HPA. (**a**) The Gt-telomere HPA strategy (see **Supplementary Methods** online). (**b**) Linearity of Gt-telomere HPA. Hybridization of acridinium ester–labeled telomere HPA probes (29-mer) with the indicated amounts of wild-type telomere 84-mer, 5′-$(TTAGGG)_{14}$-3′. Error bars, s.e.m.; $n = 3$. (**c**) Specific detection of mammalian telomere sequence by Gt-telomere HPA. Genomic DNAs were pretreated with ExoI or without ExoI before G tails were assayed. Data are mean ± range, $n = 2$. (**d**) Quantitative detection of 20-nt G tails by Gt-telomere HPA. In each experiment indicated amounts and indicated sizes of G-tail DNA were used. Error bars, s.e.m.; $n = 3$. (**e**) Detection of G tails in SiHa cell lysates. Data are mean ± range, $n = 2$.

We evaluated the sensitivity of Gt-telomere HPA, especially in terms of the minimal G-tail length that can be detected, using synthetic telomeric DNA constructs with G-tail lengths of 10–62 nt (**Supplementary Fig. 4** online). Despite the fact that the HPA probe is a 29-mer, we obtained HPA signals when we used G-tail DNAs shorter than 29-mer G-tail DNA probe (**Fig. 1d**). We predicted that this was due to the partial hybridization of telomere HPA probe to G-tail DNA as shown in **Supplementary Fig. 4**. Although 10-nt G tails were detectable and ExoI sensitive (data not shown), linearity was obtained only between 20 nt and 62 nt, indicating that Gt-telomere HPA can be used to quantitatively detect 20-nt G tails (**Fig. 1d**). To estimate how many perfect nucleotide pairs in hybrids between telomere repeats and the probe were required for resistance of acridinium ester to alkaline treatment, we hybridized mutant oligonucleotide and mutant HPA probes. We found that mismatches six bases away from the acridinium ester–labeled position did not affect HPA signal (**Supplementary Fig. 5** online).

To measure G-tail lengths in cell lysates, we lysed cell pellets from the SiHa cancer cell line in a high-salt concentration hybridization buffer that contained the detergent, lithium lauryl sulfate. Gt-telomere HPA displayed a good linearity in the range 1×10^5 to 3.5×10^6 cells (**Fig. 1e**). Thus, a sample of 5×10^5 cells is sufficient for measurement of telomeric G tails.

Next, we carried out experiments to evaluate the utility of our new method. First, we measured mean telomeric G-tail lengths in genomic DNA (**Fig. 2a**) and cell pellets (**Fig. 2b**) of normal and hTERT-infected TIG-3 human fibroblasts and of SV40-transformed TIG-3 cells at crisis. Our results (**Fig. 2**) are consistent with the previous reports that human telomeric G tails are reduced during cellular senescence[10] and at crisis in SV40-transformed cells[5]. We observed no reduction of G-tail lengths in human telomerase revere transcriptase, hTERT-expressing TIG-3 cells. Second, we examined the effect of $TERF2^{\Delta B \Delta M}$ (that encodes TERF2 lacking both N-terminal basic domain and C-terminal Myb domain), the dominant negative allele of telomere repeat binding protein 2 (*TERF2*, also known as *TRF2*) that is known to reduce the lengths of G tails[11] in SiHa cells. We found that these cells had the expected reduction in their G-tail lengths (**Fig. 2**). These findings are consistent with those of previous reports[1,2,12]. In both of these experiments, the data from cell pellets were consistent with those from purified genomic DNA (**Fig. 2**). Thus, direct measurement using cell pellets is feasible with Gt-telomere HPA. *Alu* elements are a class of short interspersed elements (SINEs) that have expanded to a copy number of more than one million elements in primate genomes. The *Alu*-HPA probe can be used to normalize the amount of total genomic

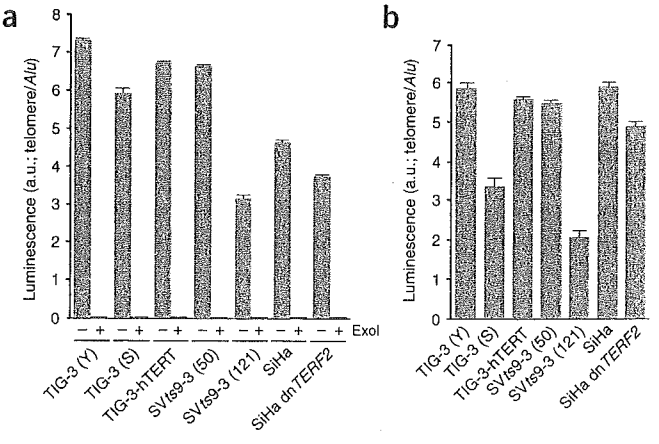

Figure 2 | Mean G-tail lengths in cultured human cells. (**a**) G-tail length measured in purified genomic DNA from human cells either with or without ExoI pretreatment expressed in arbitrary units (a.u.): ((rlu by telomere probe ÷ rlu by *Alu* probe) × 100). TIG-3(Y), normal young at 28 population doubling levels (PDLs); TIG-3 (S), senescent cells at 81 PDLs; TIG-3-hTERT, hTERT-introduced cells; SV*ts*9-3 (50), young SV40-transformed cells at 50 PDLs; SV*ts*9-3 (121), crisis stage cells at 121 PDLs; SiHa, control vector-infected; SiHa dn*TERF2*, dominant-negative allele $TERF2^{\Delta B \Delta M}$-infected. Error bars, s.e.m.; $n = 3$. (**b**) Gt-telomere HPA was performed using lysates derived from 5×10^5 cells. Data are mean ± range, $n = 2$.

DNA, even when cell numbers have been adjusted before performing the assay (**Supplementary Fig. 6** online). As the number of *Alu* sequences may vary between individuals, some variation in normalization is possible with our method. Direct measurement from lysates of 5×10^5 or fewer cells may eliminate genomic DNA loss during sample preparation. This may be an advantage when limited numbers of cells are available as occurs, for example, in clinical samples from blood inspection, fine needle aspiration or cancer cells in urine.

Although the rlu values produced by Gt-telomere HPA give only the average luminescence produced by G tails in a cell population, mean G-tail lengths can be estimated from those rlu values using an analytical curve (**Fig. 1b**) and chromosome karyotype information (**Supplementary Fig. 7** online). The estimated average size of the G tails produced by Gt-telomere HPA are comparable to those reported using other methods. Although our method can be used to estimate the difference in mean G-tail lengths between cell populations, if specific G-tail shortening in a specific chromosome was the trigger for a biological effect, then Gt-telomere HPA would not be suitable for identifying this change. Likewise, Gt-telomere HPA could not detect the known differences in G-tail lengths between leading strand and lagging strand ends[2,13]. Despite these limitations, however, Gt-telomere HPA offers other advantages such as large-scale screening. In addition, Gt-telomere HPA will have practical benefits for the basic research in cancer, aging, chromosome and telomere biology.

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Editorial Board

Growth Factors and Oncogenes in Gastrointestinal Cancers

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Keywords

Cell Adhesion Molecules
Surface ligands, usually glycoproteins, that mediate cell to cell adhesion. Their functions include the assembly and interconnection of various vertebrate systems, as well as maintenance of tissue integration, wound healing, morphogenic movements, cellular migration, and metastasis.

Cell Cycle Regulators
Proteins that regulate the cell division cycle. This family of proteins involves a wide variety of classes, including cyclin-dependent kinases, mitogen-activated kinases, cyclins, and phosphoprotein phosphatases as well as their putative substrates, such as chromatine-associated proteins, cytoskeletal proteins, and transcription factors.

Cytokines
Polypeptides secreted by inflammatory leukocytes, macrophages and lymphocytes in response to microbes and other antigens that mediate and regulate immune and inflammatory reactions. They generally act locally in a paracrine or an autocrine manner rather than in an endocrine manner.

Growth Factors
Signal molecules that act to control cell growth and differentiation in the receptor-dependent fashion. The alterations of these proteins lead to transformation and the accompanying loss in growth control. Some of the growth factors and their receptors are involved in the products of oncogens.

Oncogenes
Genes that can convert cells to cancerous growth by attacking crucial cellular machinery. They encode for growth factors, growth-factor receptors, protein kinases, signal transducers, nuclear phosphoproteins, and transcription factors. These genes are constitutively expressed after structural and /or regulatory changes, resulting in uncontrolled cell proliferation. They can be classified into viral oncogenes (v-oncogenes) and cellular oncogenes (proto-oncogenes).

Telomerase
Essential ribonucleoprotein reverse transcriptase that adds telomeric DNA to the ends of eukaryotic chromosomes. Telomerase is expressed in the testis and ovary, but repressed in normal human somatic tissues. Telomerase activity is seen in more than 90% of human cancers.

Tumor-suppressor Genes
Genes inhibit expression of the tumorigenic phenotype. They are normally involved in holding cellular growth in check. When tumor-suppressor genes are inactivated or lost, a barrier to normal proliferation is removed, leading to unregulated growth.

A large number of molecular events are involved in the development and progression of gastrointestinal carcinomas. Among them, common and distinct events of genetic and epigenetic alterations in oncogenes, tumor-suppressor genes, cell adhesion molecules, DNA repair genes, and genetic instability as well as telomerase activation are observed in esophageal, gastric, and colorectal cancers. In gastric cancer, the pattern of genetic and epigenetic alterations also differs depending on the two histological types, intestinal type or well-differentiated type and diffuse type or poorly differentiated type, indicating that there are two distinct major genetic pathways for gastric carcinogenesis.

In addition to these events, gastrointestinal cancer cells express a broad spectrum of the growth factor/cytokine receptor systems that organize complex cancer-stromal interaction, which confer cell growth, apoptosis, morphogenesis, angiogenesis, progression, and metastasis. However, these abnormal growth factor/cytokine networks also are different among esophageal, gastric, and colorectal cancers, respectively. Importantly, NF-κB activation induced by inflammation may act as a key player for induction of growth factor/cytokine network in gastrointestinal cancers.

1 Introduction

Multiple genetic and epigenetic alterations in oncogenes, tumor-suppressor genes, cell cycle regulations, cell adhesion molecules, DNA repair genes and genetic instability as well as telomerase activation are responsible for the multistep process of human gastrointestinal carcinogenesis. However, a scenario or particular combination of these alterations differs in esophageal, gastric, and colorectal cancers. Namely, common and distinct molecular events are observed in esophageal, gastric, and colorectal cancers, respectively. Moreover, two types of gastric cancer, well-differentiated or intestinal type, and poorly differentiated or diffuse-type carcinomas also exhibit a distinct pattern of genetic pathways.

Besides these genetic and epigenetic events, gastrointestinal cancer cells express a broad spectrum of growth factors, cytokine or both, including epidermal growth factor (EGF) family, transforming growth factor (TGF)-β, heparin binding (HB)-EGF, PDGF, IGF, basic fibroblast growth factor (FGF), interleukin (IL)-1α, IL-6, IL-8 and osteopontin (OPN). These growth factors and cytokines act as autocrine, paracrine, and juxtacrine modulators of the growth of cancer cells, and then organize complex interplay between cancer cells and stromal cells, which plays an important role in cell growth, apoptosis, morphogenesis, angiogenesis, progression and metastasis. Interestingly, the expression of these growth factors, cytokines or both by cancer cells is also different among esophageal, gastric, and colorectal cancers.

This article will provide an overview of the molecular machinery that underlies gastrointestinal carcinogenesis and focuses on abnormal growth factor/cytokine network in gastrointestinal cancers.

2 Genetic and Epigenetic Alterations and Abnormal Growth Factor/Cytokine Network in Esophageal Cancer

Esophageal cancer is the third most frequent gastrointestinal cancer in the world. The most recent estimates are that esophageal cancer is the sixth most common cancer in men (212 600 new cases, 4.9% of all cancers) and the ninth most common in women (103 200 new cases, 2.7% of all cancers). The two main histological types of esophageal cancer are squamous cell carcinoma (SCC) and adenocarcinoma, but SCC is the more prevalent type worldwide. The development of esophageal SCC exhibits a multistep, progressive process. An early indicator of this process is an increased proliferation of esophageal epithelial cells including basal cell hyperplasia, dysplasia, and carcinoma *in situ*. This multistep process requires the accumulation of multiple genetic and epigenetic alterations and overexpression of growth factors/cytokine receptor systems, leading to the evolution of clonal cell populations that possess growth advantages over other cells as demonstrated in the progression model of head and neck cancer. This paragraph thus will describe recent advances in molecular dissection of multistep tumorigenesis of esophageal SCC and abnormal growth factor/cytokine network that contributes to the development and progression of esophageal SCC.

2.1 Genetic and Epigenetic Alterations in Esophageal SCC

Numerous genetic and epigenetic alterations are implicated in the development and progression of esophageal SCC (Table 1). This cancer is frequently associated with loss of heterozygosity (LOH) at multiple chromosomal loci including 3p, 5q, 9p, 9q, 13q, 17p, 17q, and 18q. No significant differences have been found in the prevalence of LOH at various loci in SCC and adenocarcinoma of the esophagus.

Among these alterations, LOH and mutation of the *p53* gene at chromosome 17p13 occur at an early stage of esophageal carcinogenesis, such as dysplasia and carcinoma *in situ*. About 50% of esophageal SCC harbor mutations of the *Tp53* gene, most of which are missense mutations leading to amino acid changes within exons 5–8, which encode the entire DNA binding domain of the p53 molecule and the flanking splice sites. Considering the

Tab. 1 Genetic and epigenetic alterations found in esophageal SCC.

Genetic and epigenetic alterations	*Incidence [%]*
Tumor suppressors	
P53 LOH, mutation	40–60
APC LOH	60–70
DCC LOH	20–40
Rb LOH	40–50
BRCA1 LOH	60
3p LOH	40–100
5p LOH	62
9p LOH	45–76
9q LOH	60
13q LOH	57
14q LOH	65
17p LOH	43–65
17q LOH	62
RARβ2 loss	40–50
Cell cycle regulators	
p16 loss, mutation	45–76
Cyclin D_1 amplification	40–50
Oncogenes	
EGFR amplification	10–15
Telomerase activity	86
TERT expression	96

base substitution spectrum, G:C to T:A transversion is common in esophageal carcinoma, similar to that in carcinomas of the lung and liver. This situation is different from the finding that colorectal carcinomas frequently contain G:C to A:T transitions at CpG dinucleotides. This evidence suggests that different environmental and intrinsic factors may affect the tumorigenesis of esophageal and colorectal carcinomas. It is of interest that LOH of the *APC, DCC,* and *Rb* genes shows high frequency but these genes are very rarely or never mutated in esophageal SCC.

The retinoic acid receptor (RAR) β gene is a putative tumor-suppressor gene on chromosome 3p24, where a high frequency of LOH is found in many human cancers, including esophageal cancer. The human RARβ has three isoforms (β1, β2, β4). Overexpression of RARβ2 induces inhibition of tumor cell growth and apoptosis in human cancer cell lines including esophageal cancer cells. Moreover, Induction of RARβ2 suppresses cyclooxygenase-2 (COX2) expression in esophageal cancer cells. More importantly, DNA methylation of RARβ2 promoter CpG sites has been reported to cause the loss of RARβ2 expression in many human cancers including lung, breast, prostate, stomach, head and neck, and esophageal cancers. RARβ is expressed in 90% of normal esophageal mucosa, while it is detected in only 60% of dysplastic lesions and in 50% of SCC. These findings indicate that loss of RARβ, or more specifically, the isoformβ2, is an early event associated with esophageal carcinogenesis and the status of squamous differentiation.

p16, an inhibitor of cyclin D1/cyclin-dependent kinase, is located on chromosome 9p21. It is inactivated by 9p21LOH with *de novo* p16 promoter hypermethylation in the majority of esophageal SCC. Recent molecular analysis of precancerous laryngeal lesions suggests that loss of p16 protein is an early step toward malignant transformation in head and neck tissues. This protein forms binary complexes with CDK4 and CDK6, inhibiting their ability to phosphorylate the Rb protein. Loss of the p16 protein may bring about increased Rb phosphorylation and allow cells to enter into S-phase. In fact, we have confirmed that homozygous deletion of the *p16* gene is closely correlated with the increased expression of cyclin D1, CDK4 and phosphorylated Rb protein in esophageal SCC cell lines.

In 1989, we discovered the coamplification of *hst-1* and *int-2*, both of which are located on chromosome 11q13, in about 50% of primary tumors and in 100% of metastases of esophageal SCC. Gene amplification, however, was not accompanied by overexpression of the two genes. Subsequently, Jiang et al. found that the cyclin D1, which is located on the same locus as *hst-1* and *int-2* genes, was amplified in 32% of SCC, associated with overexpression. The amplification of the cyclin D1 is closely correlated with tumor staging, depth of tumor invasion, and metastasis. In the esophagus, 71% of SCC and 64% of adenocarcinoma are positive for increased cyclin D1 nuclear staining, indicating that overexpression of cyclin D1 is common in both types of cancer. Cyclin D1 binds to Rb protein and stimulates its phosphorylation. Hyperphosphorylation of Rb in response to overexpressed cyclin D1 may lead to uncontrolled cell cycling and increased cell proliferation.

As for oncogene activation, amplification of the EGF receptor (*EGFR*) gene occurs in 10–15% of advanced cases of

esophageal SCC, accompanied by overexpression of EGFR. The frequency of K-ras mutation is very low in esophageal SCC, whereas it takes place in 50% of sporadic colorectal carcinoma. c-*erb*B2 is amplified in esophageal adenocarcinoma but not in esophageal SCC. Recently, Inazawa's group reported that *ZASC1* encoding a Krüppel-like zinc finger protein is involved in the pathogenesis of esophageal SCC as one of the targets for 3q26 amplification. *CIAP1*, a number of the *IAP* (antiapoptotic) gene family, may also be a target for 11q22 amplification.

Telomerase, a ribonucleoprotein enzyme, is necessary for cancer cells to maintain their telomere and to become immortal. Results of a 1998 study on cell immortalization show, however, that activation of telomerase alone is not enough to immortalize certain epithelial cells, and that inactivation of the p16/Rb pathway is needed. More than 80% of gastrointestinal carcinomas exhibit high level of telomerase activity and overexpression of human telomerase reverse transcriptase (hTERT). The expression of hTERT is closely associated with activation of telomerase *in vitro* and *in vivo*. It is of interest to note that telomerase activity as well as hTERT expression is detected in about 45% of dysplasia and in 90% of SCC of the esophagus. Telomerase activation may also play a critical role in early stage of esophageal SCC.

Recently, Chen et al. reported that LOH at 13q 33–34 including *ING1*, a candidate tumor-suppressor gene, was observed in about 60% of esophageal SCC, associated with mutation as well as loss of ING1 protein. ING1, a novel growth inhibitor, cooperates directly with p53 in growth regulation by modulating the ability of p53 to act as a transcriptional activator. Genetic or epigenetic alterations in ING1 may be also involved with esophageal SCC. Sonoda et al. reported that loss of *LRP1B* (low density lipoprotein receptor–related protein 1*B*) often occurs in esophageal SCC.

These results overall indicate that accumulation of the above-mentioned genetic and epigenetic alterations is involved in the multistep carcinogenesis and progression of esophageal SCC. Inactivation of tumor-suppressor genes on 3p (ex. RARβ2) and p53, and telomerase activity may be important for converting normal stratified squamous epithelium to dysplasia. Because p16 inactivation and 9q LOH are found occasionally in mild dysplasia, but frequently in severe dysplasia and in carcinoma *in situ*, these alterations may have implications for transformation to malignancy. Amplification of cyclin D1and *EGFR* genes, inactivation of tumor-suppressor genes on 5q, 13q, and 18q, and abnormal expression of growth factor/cytokine receptor system may confer progression and metastasis of esophageal SCC. The genetic progression model of esophageal SCC (Fig. 1) is quite similar to that of head and neck SCC

2.2 Abnormal Growth Factor/Cytokine Network in Esophageal SCC

Esophageal SCC cells express a variety of growth factor/ receptor and cytokines including the EGF family, PDGF, transforming growth factor β (TGFβ), interleukin (IL)-1α and IL-6. Among them, the EGF/TGFα receptor system plays a major role in the cell growth and progression of esophageal SCC through signaling of receptor-linked tyrosine kinases.

In many cancer cells, both EGF and TGFα act as autocrine growth factors through EGFR which is encoded by the proto-oncogene *c-erbB1*.

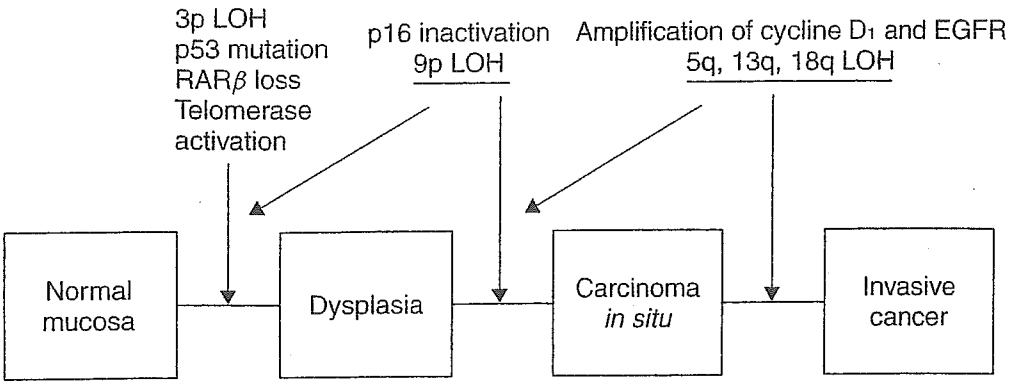

Fig. 1 Genetic progression model of esophageal SCC.

Membrane-bound TGFα binds to EGFR on the surface of contiguous cells and induces receptor autophosphorylation, leading to signal transduction, which is regarded as juxtacrine mitogenetic stimulation. In addition, EGF or TGFα induce the expression of TGFα and EGFR, thus indicating the presence of an autocrine loop of EGF/TGFα/receptor system. We have found that EGF is expressed in 30% of esophageal SCC while TGFα and EGFR are expressed in 77 and 89% of esophageal SCC, respectively. Coexpression of TGFα and EGFR is closely correlated with dysplastic progression and high grade of malignancy. The number of EGFR receptors is about 10 times higher than that in gastric carcinomas, which might be one of the reasons for the rapid growth of esophageal SCC compared to that of other carcinomas.

Esophageal SCC expresses PDGF A- and B-chains and PDGF B-receptor, suggesting the existence of a multiautocrine loop in the growth and progression of tumor cells. What is interesting is that EGF or TGFα increase the expression of PDGF A- and B-chain in esophageal SCC cell lines.

Grb7, a ligand for both EGFR and c-*erb*B2, is also implicated in esophageal SCC. Tanaka et al. found overexpression of Grb7in 44 % of the tumors and coexpression of Grb7 with EGFR or c-*erb*B-2 in 32% of the advanced cases, suggesting that Grb7 functions as an extracellular stimulus for progression of esophageal SCC.

EGF or TGFα produced by the tumor cells can make not only an autocrine loop of EGF/TGFα receptor system for tumor growth stimulation but also an induction of matrix metalloproteinases such as intestinal collagenase and stromelysin to evoke a cascade of cellular events that are involved in extracellular matrix degradation and tumor invasion. Moreover, one of the important substrates of EGFR kinase is β-catenin, a regulatory protein for cadherin. Upon phosphorylation of β-catenin, the cells are dissociated by loss of cadherin function. A phosphorylated form of β-catenin is detected in tumor cells of esophageal SCC and adenocarcinomas. β-catenin is also involved as a downstream transcriptional activator of the Wnt signaling pathway. Recently, β-catenin has been shown to induce cyclin D1 expression, suggesting that free β-catenin may be implicated in an increase in cell cycling.

In addition to phosphorylation of β-catenin, downregulation of E-cadherin and α-catenin occurs in both esophageal SCC and adenocarcinomas, while upregulation of P-cadherin takes place in

esophageal SCC. The reduced expression of α-catenin correlates more with invasion and nodal metastasis than E-cadherin reduction in esophageal SCC.

Above all, esophageal SCC exhibit multiple autocrine growth factor-receptor loops that may participate not only in cell growth but also in tumor invasion and metastasis, associated with reduction of cell adhesion molecules. Understanding the biology of esophageal cancer is indispensable to precise diagnosis and proper cancer treatment.

3 Genetic and Epigenetic Alterations and Abnormal Growth Factor/Cytokine Network in Gastric Cancer

Gastric cancer is the most common cancer worldwide and is second only to lung cancer as a cause of cancer mortality. Most recent world estimates indicate that 798 000 new cases are diagnosed and 628 000 deaths occur annually from gastric cancer. The highest incidence is still observed in Japan because of the remarkable increase in the aged population over 60 years old. Most gastric cancers arise distally from the antrum and pylorus, but about 20% involve the cardia and fundus and approximately 10% involve the stomach diffusely.

There are several histological classifications of gastric cancer. Lauren divided gastric cancer into two types, intestinal and diffuse, and the Japan Research Society for Gastric Cancer classified it into five common types. The JRSGC classification is similar to that of the World Health Organization. This article will use a two-type classification: the intestinal or well-differentiated type and the diffuse or poorly differentiated type.

The genetic and epigenetic changes found in gastric carcinoma differ depending on the histological type of gastric cancer, indicating that different carcinogenetic pathways exist for the intestinal and diffuse types of carcinomas. In addition, cancer-stromal interaction through the growth factor/cytokine receptor system, which plays a pivotal role in morphogenesis, cancer progression, and metastasis, is also different between the two types of gastric carcinomas.

Meta-analysis of epidemiological studies and animal models show that both intestinal and diffuse types of gastric cancer are equally associated with *Helicobacter pylori* (*H. pylori*) infection. However, *H. pylori* infection may play a role only in the initial steps of gastric carcinogenesis. Differences in *H. pylori* strain, patient age, exogenous or endogenous carcinogens and genetic factors such as DNA polymorphism and genetic instability may be implicated in two distinct major genetic pathways for gastric carcinogenesis.

The next paragraph will describe the genetic pathways of the two types of gastric cancer and the abnormal growth factor/cytokine network that organizes remarkably complex interplay between cancer cells and stroma cells in gastric cancer.

3.1 Genetic and Epigenetic Alterations in Gastric Cancer

Genetic and epigenetic alterations in oncogenes, tumor-suppressor genes, cell-cycle regulators, cell adhesion molecules, DNA repair genes, and genetic instability as well as telomerase activation, are responsible for tumor genesis and progression of gastric cancer (Tables 2 and 3; Figs. 2 and 3). Among them, inactivation of various genes including *p16*, *hMLH1*, cadherin1 (*CDH1*),

Tab. 2 Genetic and epigenetic alterations found in intestinal and diffuse types of gastric cancer (1).

Genetic and epigenetic alterations	*Incidence of cases with indicated alterations [%]*	
	Intestinal type	*Diffuse type*
Tumor suppressors		
p53 LOH, mutation	60	75
p73 LOH	53[a]	24
APC LOH, mutation	40–60	0
DCC LOH	50	0
LOH of Chr. 1q	44	0
LOH of Chr. 7q	53	33
LOH of Chr. 17q	0	40[b]
Loss of pS2 expression	49	31
Loss of RARβ2	50	73[c]
Loss of RUNX3	37	40
Cell cycle regulators		
Cyclin E amplification	33	7
Cyclin E overexpression	26	27
CDC25B overexpression	33	73
Loss of p16 expression	50[d]	10
Loss of p27 expression	46	69

[a] Preferentially found in foveolar-type adenocarcinoma.
[b] Preferentially found in patients younger than 35 years of age.
[c] $p = 0.0335$; Fisher's exact test.
[d] $p = 0.0023$; Fisher's exact test.

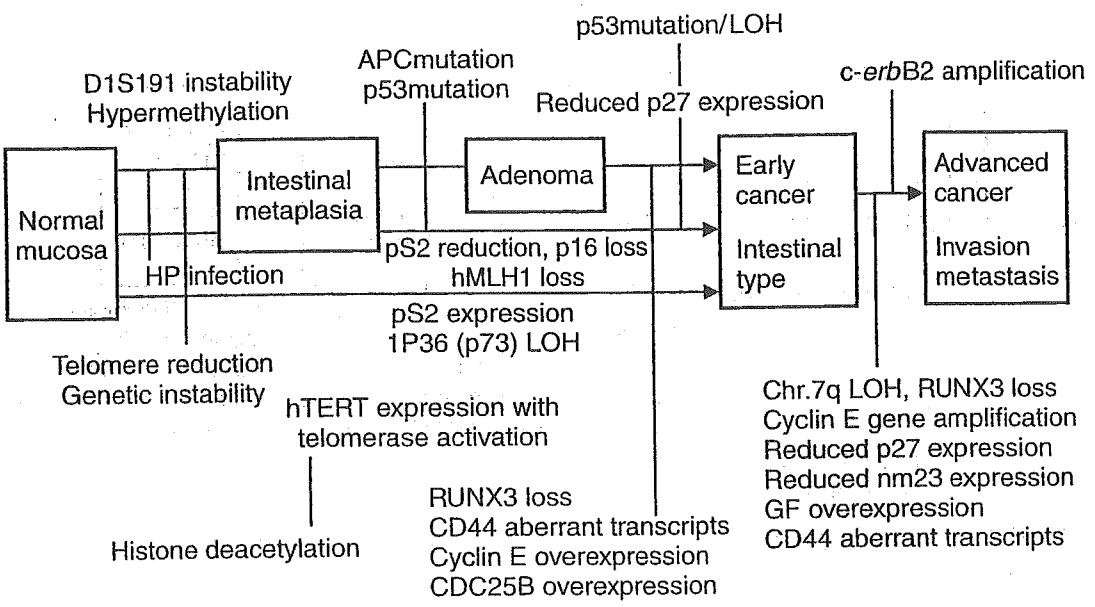

Fig. 2 Multiple genetic and epigenetic alterations during human gastric carcinogenesis (intestinal type).

Tab. 3 Genetic and epigenetic alterations found in intestinal and diffuse types of gastric cancer (2).

Genetic and epigenetic alterations	*Incidence of cases with indicated alterations [%]*	
	Intestinal type	*Diffuse type*
Oncogenes		
K-ras mutation	10	0
c-met amplification	19	39
K-sam amplification	0	33
c-*erb*B2 amplification	20	0
Adhesion molecules		
E-cadherin, mutation	0	50
Loss of CDH1	55	79[b]
CD44 aberrant transcript	100	100
Microsatellite instability	20–40	20–70[a]
(hMLH1 methylation)	(5–20)	(0)
Histone deacetylation	61	82
Telomere/Telomerase		
Telomere reduction	62	53
Telomerase activity	100	90
TERT expression	100	86

[a] Preferentially found in patients younger than 35 years of age.
[b] $p = 0.0175$; Fisher's exact test.

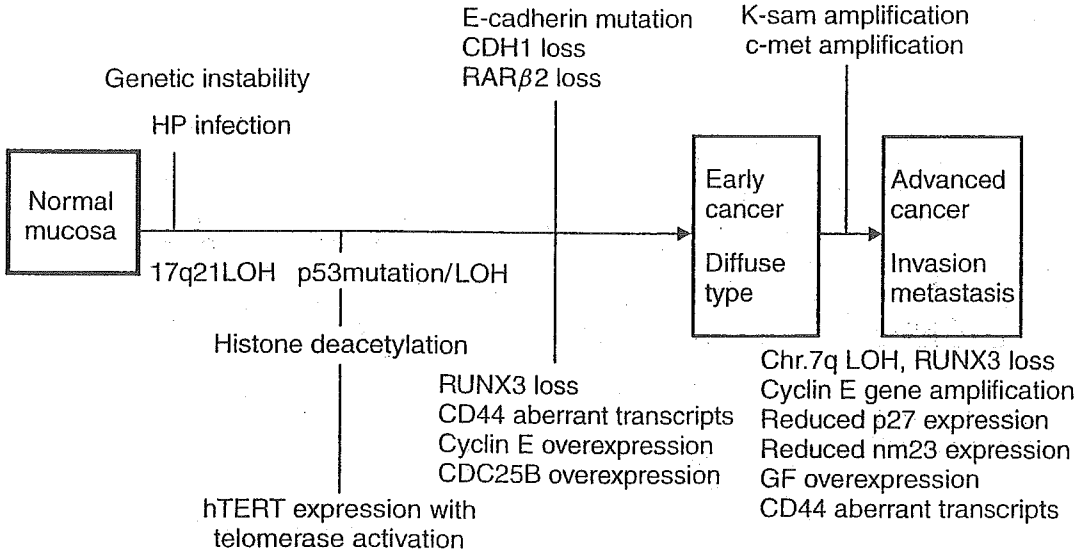

Fig. 3 Multiple genetic and epigenetic alterations during human gastric carcinogenesis (diffuse type).

RARβ2, *pS2* and *RUNX3* by DNA methylation is involved in two distinct major genetic pathways of gastric cancer. Hypermethylation of the p16 and of hMLH1 promoters is preferentially associated with intestinal type, whereas concordant hypermethylation of the CDH1 and RARβ2 promoters predominantly occurs in diffuse type gastric carcinoma. Loss of RUNX3 and pS2 expression by promoter methylation is a common event in both types of gastric carcinoma. The scenario of these epigenetic alterations indicates that there are at least two types of CpG island methylator phenotypes in intestinal and diffuse types of gastric cancer. Recently, Cho et al. reported that promoter hypomethylation of a novel cancer/testis antigen gene CAGE was found in 35% of chronic gastritis and in 78% of gastric cancer.

In addition to promoter hypermethylation or hypomethylation, acetylated histone H4 is obviously reduced in the majority of gastric carcinoma. Histone H4 is progressively deacetylated from the early stage (precancerous lesions) to the late stage (invasion and metastasis) in gastric carcinogenesis. Since there is no difference in the level of acetylated histone H4 between the intestinal and diffuse types of gastric cancer, histone H4 deacetylation is a common event in both types of gastric cancer.

In the multistep process of intestinal type carcinogenesis, the genetic pathways can be divided into three subways: an intestinal metaplasia → adenoma → carcinoma sequence, an intestinal metaplasia → carcinoma sequence and *de novo*. Infection with *H. pylori* may be a strong trigger for hyperplasia of hTERT-positive "stem cell" in intestinal metaplasia. Genetic instability and hyperplasia of hTERT positive stem cells precede replication error at the D1S191, DNA hypermethylation at the D17S5 locus, pS2 loss, RARβ2 loss, RUNX3 loss, CD44 abnormal transcripts and p53 mutation, all of which accumulate in at least 30% of incomplete intestinal metaplasia. All of these epigenetic and genetic changes are common events in intestinal type gastric cancer. An adenoma → carcinoma sequence is found in about 20% of gastric adenoma with APC mutations. In addition to these events, p53 mutation and LOH, RUNX3 loss, reduced p27 expression, cyclin E overexpression and abnormal transcript of c-met allow malignant transformation from the above precancerous lesions to intestinal type gastric carcinoma (Fig. 2). DCC loss, APC mutations, 1qLOH, p27 loss, reduced TGFβ receptor, reduced *nm23* and *c-erbB2* gene amplification are frequently associated with an advanced stage of intestinal-type gastric carcinoma. The *de novo* gastric carcinoma involves LOH and abnormal expression of *p73* gene that is responsible for the development of foveolar-type gastric cancers with pS2 expression.

On the other hand, LOH at chromosome 17p, mutation or LOH of p53, LUNX3 loss, and mutation or loss of E-cadherin are preferentially involved in the development of diffuse type gastric carcinoma. These events may occur simultaneously or in the relatively short term in superficial gastritis induced by *H. pylori* infection. In addition to these alterations, gene amplification of *K-sam* and *c-met*, RUNX3 loss, 7q LOH, cyclin E gene amplification, p27 loss as well as reduced nm23 confer progression, and metastasis, frequently associated with productive fibrosis. Mixed gastric carcinomas composed of intestinal and diffuse components exhibit some but not all of the molecular events described

so far for each of the two types of gastric cancer (Fig. 3).

Several proto-oncogenes, including *c-met*, *K-sam*, and *c-erbB2*, are frequently activated in gastric cancer. The amplification of the *c-met* gene encoding a receptor for hepatocyte growth factor/scatter factor (HGF/SF) is found in 19% of intestinal type and 39% of diffuse type gastric cancers. Most of gastric carcinomas express two different c-met transcripts, one of 7.0 kb and another of 6.0 kb. Expression of the 6.0 kb c-met transcript correlates well with tumor staging, lymph node metastasis, and depth of tumor invasion. The *K-sam* gene has at least four transcriptional variants. Type II transcript, which is expressed only in carcinoma cells, encodes a receptor for keratinocyte growth factor (KGF). *K-sam* is preferentially amplified in 33% of advanced diffuse or scirrhous type gastric cancer. But *K-sam* gene amplification is never seen in esophageal or colorectal carcinomas. In contrast to *K-sam*, *c-erbB2* is preferentially amplified in 20% of intestinal type gastric cancer but not in diffuse type gastric cancer. Overexpression of *c-erbB2* associated with gene amplification confers a poor prognosis and liver metastasis. The amplification of *c-erbB1* (EGFR) and *c-erbB3* is seen in 3 and 0%, respectively, of gastric cancer.

RUNX3, a Runt domain transcription factor involved in TGFβ signaling, is a candidate tumor-suppressor gene localized in 1p36, a region commonly deleted in a variety of human cancers, including gastric cancer. *RUNX* gene family is composed of three members, *RUNX1/AML1*, *RUNX2* and *RUNX3*, and encodes the DNA binding(α) subunits of the Runt domain transcription factor polyomavirus enhancer-binding protein 2 (PEBP2)/core-binding factor (CBF), which is a heterodimeric transcription factor. All three *RUNXs* play important roles in both normal developmental processes and carcinogenesis. *RUNX1*, which is required for definitive hematopoiesis, is the target of chromosome translocations in leukemia. *RUNX2*, which is essential for osteogenesis, is mutated in the human disease cleidocranial dysplasia. *RUNX3* is necessary for the suppression of cell proliferation of gastric epithelium, neurogenesis of the dorsal root ganglia, and T-cell differentiation. The gastric epithelium of RUNX3 knockout mice shows hyperplasia, reduced rate of apoptosis, and reduced sensitivity to TGFβ1, suggesting that the tumor-suppressor activity of RUNX3 operates downstream of the TGFβ signaling pathway.

Recent studies on RUNX3 methylation in human cancers demonstrated that loss of RUNX3 by hypermethylation of the promoter CpG island was detected not only in 64% of gastric cancer but also in 73% of hepatocellular carcinoma, 70% of bile duct cancer, 75% of pancreatic cancer, 62% of laryngeal cancer, 46% of lung cancer, 25% of breast cancer, 23% of prostate cancer, 12% of endometrial cancer, 2.5% of uterine cervical cancer, and 5% of colon cancer. The RUNX3 methylation is especially frequent in cancers from tissues of a foregut origin. Interestingly, the RUNX3 methylation is found in 8% of chronic gastritis, 28% of intestinal metaplasia, and 27% of gastric adenoma, but not in chronic hepatitis B. These findings suggest that RUNX3 is a target for epigenetic gene silencing in gastric carcinogenesis.

Cell adhesion molecules may also work as tumor suppressors. Mutations in the E-cadherin gene occur preferentially in 50% of diffuse type gastric carcinoma. E-cadherin mutations affecting exons 8 or 9 induce the scattered morphology, decreased cellular adhesion and increased

cellular motility of diffuse gastric cancers. The mutations are even detected in intramucosal carcinoma. E-cadherin germline mutations responsible for hereditary diffuse gastric cancer (HDGC) have been reported since 1998, but their frequency is extremely rare. The β- and γ-catenin mutations but not E-cadherin mutations bring about constitutive Tcf transcriptional activity in gastric and pancreatic cancer cells. As mentioned in esophageal SCC, cross-talk between β-catenin and receptor tyrosine kinases including c-met, EGFR and c-*erb*B2 is found in gastric cancer cells, leading to diffuse spreading or scattering of gastric cancer cells through enhanced Wnt signaling pathway. These results indicate that genetic and epigenetic alterations in E-cadherin and catenins are involved in the development and progression of diffuse and scirrhous-type gastric cancer.

As to alterations in cell-cycle regulators, the cyclin E gene is amplified in 15 to 20% of gastric carcinomas that are associated with overexpression. Gene amplification or overexpression of cycline E, or both cause aggressiveness and lymph node metastasis. Cyclin D1 gene amplification, on the other hand, is exceptional in gastric cancer but frequently occurs in esophageal SCC. p27, a member of the cip/kip family of CDK inhibitors, binds to a wide variety of cyclin/CDK complexes and inhibits kinase activity. We have found that growth suppression of interferon-β is associated with the induction of p27 in the gastric cancer cell line TMK-1. More importantly, reduction in p27 expression is frequently observed in advanced gastric cancers while it is well preserved in 90% of gastric adenomas and 85% of early cancers. Reduced expression of p27 significantly correlates with tumor invasion and nodal metastasis. The expression of p27 in gastric cancer is inversely correlated with the expression of cyclin E. Loss of p27 expression and gain of cyclin E promotes progression and metastasis of gastric cancer.

Reduction in p27 occurs at posttranslational levels, resulting from ubiquitin mediated proteosomal degradation rather than genetic abnormalities.

An important downstream target of cyclin/CDKs at G1/S transition is a family of E2F transcription factors. Gene amplification of E1F-1 is seen in 4% of gastric cancers and in 25% of colorectal cancers. Overexpression of *E2F* is detected in 40% of primary gastric carcinomas. In addition, E2F and cyclin E tend to be coexpressed in gastric cancer, whereas 70% of gastric cancers show lower levels of E2G-3 expression than corresponding normal mucosa. These results overall suggest that gene amplification and abnormal expression of the *E2F* gene may permit the development of gastric cancer.

3.2 Factors Associated with Increased Incidence of Gastric Cancer

Three major factors, including environmental factors, host factors and genetic factors, cooperatively affect the genesis of gastric cancer. Of these, environmental factors are the most important, as diet and cigarette smoking are primary offenders; in particular, the presence of carcinogens such as *N*-nitroso compounds and benzo[α]pyrene is directly linked to carcinogenesis. The mutation spectrum of the *p53* gene is different between intestinal type and diffuse type gastric cancers, as p53 mutation at A:T sites are common in intestinal type carcinoma whereas GC $\rightarrow$ AT transitions are predominant in diffuse type carcinoma. Carcinogenic *N*-nitrosoamines, which cause mainly GC $\rightarrow$ AT base substitutions, are found in many

foods and can also be produced from amines and nitrates in the acidic environment of the stomach.

As for host factors, meta-analysis of relationship between *H. pylori* infection and gastric cancer has indicated that *H. pylori* infection is associated with a twofold increased risk of gastric cancer. Younger *H. pylori* infected patients have a higher relative risk of gastric cancer than older patients. *H. pylori* infection is equally associated with intestinal-type and diffuse-type gastric cancers. In fact, the observations in a Mongolian gerbil model of stomach carcinogenesis show that *H. pylori* infection promotes stomach carcinogenesis induced by chemical carcinogens, and that histological types of gastric carcinoma may depend on the concentration of chemical carcinogens rather than *H. pylori* infection. Eradication of the bacteria evidently decreases the incidence of gastric cancer in the Mongolia gerbil model.

H. pylori infection produces reactive oxygen and nitrogen species that cause DNA damage, followed by chronic gastritis and intestinal metaplasia. Goto et al. reported that the expression of inducible nitric oxide synthase (iNOS) and nitrotyrosine in the gastric mucosa was significantly high in *H. pylori* infected patients who developed gastric cancer at least two years after the initial biopsies. These findings suggest that high production of iNOS and nitrotyrosine may participate in gastric carcinogenesis.

Not only can *H. pylori* activate NF-κB in gastric epithelial cells, but activated NF-κB activates the transcription of IL-1, IL-6, IL-8, TNF-α, and cyclooxygenase-2 (Cox-2). Successful eradication of *H. pylori* leads to downregulation of Cox-2 in the epithelial and stromal cells. High expression of Cox-2 mRNA, protein, and enzymatic activity is observed in the tumor cells of intestinal type gastric cancer. Loss of Cox-2 promoter methylation may enhance Cox-2 expression and promote gastric carcinogenesis associated with *H. pylori*.

Strain of *H. pylori* and genetic factors play a critical role in susceptibility to stomach carcinogenesis. Prinz et al. reported that CagA+/VacAs1+ strains of *H. pylori* that are blood-group antigen-binding adhesion (BabA2)-positive are associated with activity or chronicity of gastritis. Adherence of *H. pylori* via BabA2 may play a key role for efficient delivery of VacA and CagA. Moreover, Hatakeyama's group has recently shown that CagA binds an Src homology 2 (SH2)-containing tyrosine phosphatase SHP-2 in a tyrosine phosphorylation-dependent manner and stimulates the phosphatase activity of SHP-2. In addition, they found that prevalent CagA protein in East Asian countries are significantly more potent in binding SHP-2 and in inducing cellular morphological changes than are CagA proteins of Western isolates. Differences in the biological activity of Western and East Asian CagA protein, which are determined by variation in the tyrosine phosphorylation sites, may underlie the different incidences of gastric cancer in these two geographic areas. Regarding the development of gastric mucosa-associated lymphoid tissue (MALT) lymphoma, heat-shock protein 60 kDa (hsp60) of *H. pylori* is an important antigen in the pathogenesis of MALT lymphoma.

In addition to *H. pylori* strains, DNA polymorphism including HLA, MUC1, T-cell helper 1 and IL-1β has been reported to be associated with an increased risk of both atrophic gastritis induced by *H. pylori* and gastric cancer. In addition, El-Omar et al. reported that proinflammatory genotypes of tumor necrosis factor α and IL-10 are associated with increased risk