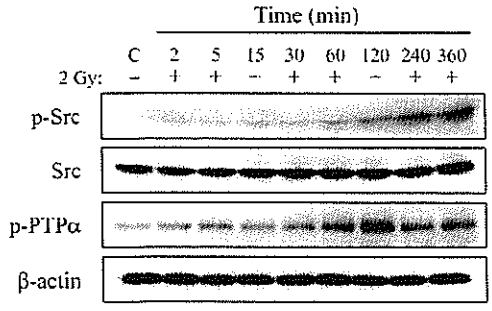

Fig. 6. Src and PTPα become phosphorylated in response to 2 Gy irradiation. MDA-MB-468 cells were irradiated with 2 Gy and incubated for 2–360 min at 37 °C. Whole cell lysates were then prepared and immunoblotted for phospho-Src (Tyr416), Src, phospho-PTPα (Tyr789), and β-actin.

Discussion

In the present study, IR activated PTPα, Src, SHP-2, EGFR, and ERK1/2. Src inhibitor PP2 suppressed activation of SHP-2 and ERK1/2, and EGFR inhibitor AG1478 suppressed activation of ERK1/2. These findings suggest that Src is an upstream signal for IR-induced ERK1/2 activation and are consistent with abundant evidence indicating that hydrogen peroxide evokes Src-dependent EGFR transactivation [20,21].

IR has been shown previously to activate EGFR, but the mechanism of the activation is not clear [1–3,22]. Our results indicate that IR activates EGFR via a mechanism that is distinct from EGFR autophosphorylation (Figs. 3 and 4). Ligand-independent transactivation of EGFR has been described with respect to a number of diverse stimuli including G-protein-coupled receptors, cytokines, and cellular stress [23]. The data presented here are in agreement with reports of H_2O_2-induced EGFR transactivation [20]. In that study, Src-activation by hydrogen peroxide induced EGFR-transactivation without phosphorylation of Tyr1173, an autophosphorylation site of the activated EGFR [20]. Our data indicate that IR induced EGFR tyrosine phosphorylation but does not involve EGFR phosphorylation at Tyr845, Tyr992, Tyr1045, and Tyr1068, which might be incompatible with EGFR activation (Fig. 3B). As Chen et al. [20] pointed out in their report, undescribed actions of AG1478 against Src kinase activity might be considered as potential explanations for these observations.

An important mechanism for regulation of Src tyrosine kinase activity is through control of its phosphorylation status [24]. The two major phosphorylation sites are an activating Tyr416 and an inhibiting Tyr527 [19,24]. PTPα has been demonstrated to dephosphorylate Src at Tyr527 and activate Src kinase activity [25]. In the present study, PTPα became activated 1–6 h after IR as indicated by its phosphorylation on Tyr789, which might be a cause of the Src activation observed 1–6 h after IR (Fig. 6). It is still unknown how PTPα is regulated during physiological cellular signaling process. Protein kinase C is demonstrated to phosphorylate PTPα, which results in stimulation of its phosphatase activity [26,27]. Another mechanism by which regulates PTPα activity is dimerization that inhibits PTPα activity [27]. However, no physiological mechanism has been demonstrated through which the formation of the dimers can occur.

SHP-2 is a well-known positive effector of EGFR signaling [16,28,29]. In the present study, SHP-2 became activated 2 min and 1–6 h after irradiation and this activation was inhibited by PP2 (Fig. 5). The time course of IR-induced SHP-2 activation partially corresponds with that of IR-induced ERK1/2 activation. These data suggest that IR-induced ERK1/2 activation involves Src-dependent SHP-2 activation.

ERK1/2 activation observed 2–5 min after irradiation is suggested to be mediated through Src because PP2 inhibited the activation (Figs. 3B and 5B). However, Src became activated only 1–6 h after irradiation without apparent activation at 2–5 min post irradiation as indicated by its phosphorylation at Tyr416 (Fig. 6). One possible mechanism of this discrepancy is the Src activation by SHP-2 without increasing Src phosphorylation at Tyr416. Walter et al. [30] reported that SHP-2 activates Src by a non-enzymatic mechanism without significant changes in phosphorylation status of Src and that a phosphatase-inactive mutant of SHP-2 can also activate Src. Src activates SHP-2 by phosphorylating SHP-2 tyrosine residue with physically association with SHP-2 [17,18]. In this study, PP2 inhibited enhanced phosphorylation of SHP-2 observed 2 min after irradiation, which might indicate Src activation at 2 min after irradiation without increased phosphorylation at Tyr416. Another potential explanation for inhibition of IR-induced ERK1/2 activation by PP2 without increased Src phosphorylation at Tyr416 is undescribed action of PP2 against unknown kinase activities that mediate IR-induced ERK1/2 activation.

In summary, our data suggest that IR activates Src/SHP-2. This Src/SHP-2 activation mediates EGFR transactivation that causes Ras/Raf/ERK signaling cascade activation. However, it remains to be elucidated how Src and SHP-2 become activated in response to IR.

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Recent Advances in Molecular Pathobiology of Gastric Carcinoma

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Introduction

Cancer is a chronic proliferative disease with multiple genetic and epigenetic alterations, namely, disease with altered gene expression. Integrated research in molecular pathology over the past 15 years has uncovered the molecular mechanism of the development and progression of gastric cancer [1–5]. Multiple genetic and epigenetic alterations involve inactivation of tumor suppressor genes, activation of oncogenes, abnormalities of DNA repair genes, cell-cycle regulators, cell adhesion molecules, growth factors/receptors, matrix metalloproteinases, and so on. Gastric carcinoma is histologically classified into two types, well-differentiated and poorly differentiated types, and the former can be further classified into those with gastric and intestinal phenotypes. Some of these alterations occur commonly in both well-differentiated and poorly differentiated types whereas some differ depending on the histological types or mucin phenotypes. Recent advances in genomic science have enabled revealing the molecular mechanism of stomach carcinogenesis more in detail; these include global analysis of gene expression by microarray or other techniques and study of the association of genetic polymorphism with cancer risk. A better knowledge of the molecular bases of gastric cancer may lead to new approaches to diagnosis, treatment, and prevention.

This chapter presents an overview of the classical pathway of molecular stomach carcinogenesis, mechanism of epigenetic alterations, importance of genetic polymorphism, search for novel genes specific in gastric carcinoma through global analysis of gene expression, and the clinical implications.

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TABLE 1. Footprint of molecular research on gastric cancer

Cancer in general		Gastric carcinoma	
1911	Rous sarcoma virus	1983	*Helicobacter pylori*
1914	Fijinami sarcoma virus		
1953	DNA double helix structure		
1956	Viral transformation	1984	c-*myc* amplification
1969	Oncogene theory	1985	Establishment of TMK-1
	Normal phenotype by cell fusion	1986	EGF overexpression
1970	Reverse transcriptase		H-*ras* altered expression
1971	Knudson's two-hit theory	1986	Identification of *HST-1*
1972	Epidermal growth factor (EGF)	1988	EGFR overexpression
	Apoptosis		*HER-2*/c-*er*bB2 amplification
1973	DNA transfection	1990	K-*sam* amplification
1975	Southern blot analysis	1991	Loss of E-cadherin
1976	Proto-oncogene c-*src*		Multiple loss of heterozygosity (LOH)
1979	Transformation by cellular DNA	1992	*p53*/*APC* mutations
	c-*src* encodes tyrosine kinase		c-*met* amplification
1982	Human H-*ras* oncogene		Cancer–stromal interaction
1983	Polymerase chain reaction (PCR) method	1993	Genetic changes in intestinal metaplasia
	Platelet-derived growth factor (PDGF) as c-*sis*, EGFR as v-*erb*B		Interleukin 1 (IL-1) as an autocrine growth factor
1986	*Rb* as a tumor suppressor gene		Molecular diagnosis
	Transcription factor Sp1	1994	Microsatellite instability in multiple cancer
1987	Cell adhesion molecule E-cadherin	1995	Increased telomerase activity

1988 Vogelstein's model for colon cancer
1989 *p53* as a tumor suppressor
1990 Cell-cycle regulator $p34^{CDC2}$
Microsatellite assay
Gene therapy for melanoma
1991 *APC* as a causative gene for famalial adenomatous polyposis (FAP)
Angiogenesis: VEGF
1993 *hMSH2* as a causative gene for hereditary nonpolyposis colorectal carcinoma (HNPCC)
p53 as transcription factor for p21
1994 TRAP assay for telomerase
1995 DNA microarrray technology
Serial analysis of gene expression
CpG island methylation of p16
1996 Laser capture microdissection
1997 Histone deacetylation
Human telomerase reverse transcriptase (hTERT)
1999 DNA demethylase
Chrosome 22 whole genome sequence
2001 Draft sequence of human genome
2003 Complete sequence of human genome

Era of Post-Genome and Genomic Medicine

Cyclin E gene amplification
1996 Reduced p21 expression
Microsatellite instability in precancerous lesion
1997 Reduced p27 expression
1998 E-cadherin germline mutation
hTERT expression
IL-8 and VEGF expression
1999 *hMLH1* hypermethylation
p73 genomic imbalance
2000 Hypoacetylation of histones
2002 23040 gene expression profile by microarray
2004 Serial analysis of gene expression (SAGE) libraries of gastric cancer

Overview of the Classical Pathway of Molecular Stomach Carcinogenesis

Footprint of Molecular Research on Gastric Carcinoma

We have learned from the footprint of cancer research that the history of cancer research is a repetition of establishment of hypothesis, development of new technologies, and discovery of novel findings (Table 1). For instance, Todaro and Huebner [6] hypothesized the oncogene theory in 1969 and Knudson [7] proposed the two-hit theory in 1971. After several years, methods of DNA transfection, Southern blotting, and polymerase chain reaction (PCR) amplification were developed and enabled them to verify and identify c-*src* as an oncogene and *Rb* as a tumor suppressor gene. Microarray is a powerful technique to reveal gene expression profiles of individual cancers. As of April 2003, the human genome sequence has been completed, and this is now the era of postgenome sequence and genomic medicine.

The history of molecular research on gastric carcinoma began only 20 years ago when c-*myc* amplification was found in primary gastric carcinoma in 1984 [8]. The first oncogene of gastric carcinoma, *HST-1*, was isolated from a primary gastric cancer in 1986 in the National Cancer Center in Tokyo [9]. In the late 1980s and 1990s, extensive analyses of molecular pathogenesis had been performed and the role and significance of novel genes and molecules, identified in other tumors or systems, had been clarified in gastric carcinoma with minimal time lag [4]. Examples include epidermal growth factor (EGF), EGF receptor (EGFR), E-cadherin, *p53*, cyclin E, $p27^{Kip1}$, human telomerase reverse transcriptase (*hTERT*), and *hMLH*1. The importance of DNA methylation and genetic instability during stomach carcinogenesis was also proved. In 1993, a routine system of molecular diagnosis on pathology specimens was established and this useful information was given to clinics [2,10]. Furthermore, the molecular mechanism of cancer–stromal interaction and genetic changes in intestinal metaplasia was explored, and the HGF/c-*met* system and mutations of *p53* and *APC*, respectively, were found to be involved [4]. Recently, dissection of gene expression profiles has been carried out using miroarray or other technology, and vast amounts of information regarding carcinogenesis, biological behavior, and chemosensitivity have been obtained, information that is directly connected with diagnosis and treatment.

Outline of Molecular Stomach Carcinogenesis

A variety of genetic and epigenetic alterations occur during multistep stomach carcinogenesis (Fig. 1) [1–5]; these include activation of oncogenes and growth factors/receptors, inactivation of tumor suppressor genes, DNA repair genes, and cell adhesion molecules, and abnormalities of cell-cycle regulators. Genetic alterations found in gastric carcinoma are gene amplification, point mutation, and loss of heterozygosity, whereas representative epigenetic changes are gene silencing by DNA methylation and overexpression at the transcriptional level [5]. Some alterations are found in both well- and poorly differentiated types, and others are unique depending on the histological type. The former may confer development of cancer whereas the latter may participate in tumor morphogenesis and biological behavior. Genetic

polymorphism predisposes to an endogenous cause and alters cancer susceptibility. Genetic instability, cytosine p guanine (CpG) island methylation, telomerase activation, and *p53* mutation commonly participate in the early steps of stomach carcinogenesis. Amplification and overexpression of the c-*met* and cyclin E genes are frequently associated with the advanced stage. Reduced expression of $p27^{Kip1}$ participates in both development and progression of gastric carcinoma. Overexpression of growth factors/cytokines confers progression through multiple autocrine loops. On the other hand, K-*ras* mutations, *HER*-2/c-*erb*B2 amplification, and *APC* mutation preferentially occur in the well-differentiated type. Precancerous lesions such as intestinal metaplasia and adenoma share alterations similar to those of the well-differentiated carcinomas. Loss of heterozygosity (LOH) of the *p73* gene occurs specifically in well-differentiated gastric carcinomas with foveolar epithelial phenotype. Inactivation of cadherins and catenins and amplification of the K-*sam* and c-*met* are frequently associated with poorly differentiated or scirrhous-type carcinomas.

Telomeric Repeats and Telomerase

The DNA sequence at telomeres consists of tandem repeats of TTAGGG, which protects chromosome ends from recombination and fusion and stabilizes the chromosome structure. Maintenance of the telomere by telomerase activation induces cellular immortalization [11]. Strong telomerase activity associated with hTERT expression is present in a majority of gastric carcinomas regardless of histological type and tumor staging [4]. Some intestinal metaplasia and adenomas express telomerase acvitity at certain levels. Telomerase activity is found in half of gastric adenomas at a level of activity about 10% of that in gastric carcinomas [12]. Hyperplasia of epithelial "stem cells" expressing hTERT and telomerase activity in precancerous lesion may be triggered by *Helicobacter pylori* (*H. pylori*) infection.

PINX1, a telomeric-repeat binding factor (TRF)1-binding protein, binds hTERT and inhibits its activity directly [13]. Reduced expression of PINX1 is detected in 70% of gastric carcinomas that show higher telomerase activity [13]. LOH of *PINX1* locus (8p23) is found in 33% of gastric carcinoma and is correlated significantly with reduced PINX1 expression. There are cases with reduced PINX1 expression but without LOH. Treatment with histone deacetylase inhibitor (HDAC) induces PINX1 expression, enhances histone H4 acetylation, and inhibits telomerase activity in gastric carcinoma cell lines. Therefore, reduced expression of PINX1 by LOH of PINX locus and hypoacetylation of histone H4 cause telomerase activation, resulting in cancer development.

POT1, a telomere end-binding protein, is proposed not only to cap telomeres but also to recruit telomerase to the ends of chromosomes [14]. POT1 expression levels are significantly higher in gastric carcinomas of advanced stage, and downregulation is frequently observed in those of early stage [14]. Reduced expression of POT1 is associated with telomere shortening and decreased telomerase activity. Inhibition of *POIT1* by antisense oligonucleotides increases telomere shortening, inhibits telomerase activity, and increases anaphase bridging, a sign of telomere dysfunction. Therefore, POT1 may play an important role in regulation of telomere length and that inhibition of POT1 may induce telomere dysfunction. Changes in POT1 expression levels may be associated with development and progression of gastric carcinoma.

Microsatellite Instability

Genomic instability is broadly classified into microsatellite instability associated with mutator phenotype and chromosome instability recognized by gross chromosomal abnormalities. A defect in DNA mismatch repair (MMR) is responsible for hereditary nonpolyposis colorectal carcinoma (HNPCC). Target genes for microsatellite instability (MSI) include *TGFBRII*, *IGFIIR*, *BAX*, *hMSH3*, *hMSH6*, and *MBD4* [4]. MSI or genetic instability causes accumulation of genetic alterations and participates in pathogenesis of sporadic gastric carcinomas as well [4]. The frequency of MSI is estimated to be about 30% of gastric carcinoma; the frequency is especially high in well-differentiated gastric carcinoma of foveolar phenotype with papillary morphology. Some intestinal metaplasias and adenomas also show MSI, and these should be considered "true precancerous lesions." Another important aspect of genetic instability is that multiple primary cancers frequently display MSI. Representative reports demonstrating the relation between MSI and tumor multiplicity are shown in Table 2. Although the frequency of MSI differs depending on the number and site of microsatellites, all show that the frequency of MSI is significantly higher in cases with multiple primary cancers. This finding indicates that the detection of MSI in a cancer may serve as a good molecular marker for the assessment of the risk of a second cancer in the same patient. CpG island hypermethylation of *hMLH1* and loss of expression is the main mechanism of MSI in sporadic gastric carcinoma [15].

Cell-Cycle Regulators

Cell-cycle checkpoints are regulatory pathway that control cell-cycle transitions and ensure that DNA replication and chromosome segregation are completed with high fidelity. The checkpoints also respond to damage by arresting the cell cycle to provide time for repair. Imbalance in cell-cycle regulators results in genomic instability and unbridled cell proliferation and is implicated in stomach carcinogenesis [2,4]. Table 3 shows representative abnormalities of cell-cycle regulators found in gastric carcinoma. The cyclin E gene is amplified in 15%–20% of gastric carcinoma, and the over-

TABLE 2. Representative reports of Microsatellite instability (MSI) and multiple primary gastric carcinomas

Multiple vs. solitary		MSI cases	Reference
Early gastric cancer	Multiple cancer Solitary cancer	21/63 (33%) 3/39 (8%)	Takahashi H, Endo T, Yamashita K, et al. (2002) Int J Cancer 100:419–424
Synchronous gastric cancer + adenoma	Multiple cancer Solitary cancer	9/18 (50%) 14/149 (9%)	Lee HS, Lee BL, Woo DK, et al. (2001) Int J Cancer 91:619–624
Gastric cancer	Multiple cancer Solitary cancer	11/14 (79%) 5/24 (21%)	Nakashima H, Honda M, Inoue H, et al. (1995) Int J Cancer 64:239–242
Gastrointestinal and biliary cancer	Multiple cancer Solitary cancer	34/38 (89%) 19/174 (11%)	Horii A, Han JHJ, Shimada M, et al. (1994) Cancer Res 54:3373–3375

TABLE 3. Abnormalities in cell-cycle regulators found in gastric carcinoma

Cell-cycle regulators	Method[a]	Incidence	Role[b]	References
CDC2 high kinase activity	Kinase	92%	D	Yasui W, Ayhan A, Kitadai Y et al. (1993) Int J Cancer 53:36–41
Cyclin E gene amplification	Southern	16%	P	Akama Y, Yasui W, Yokozaki H, et al. (1995) Jpn J Cancer Res 86:617–621
Cyclin E overexpression	IHC	27%	D/P	Yasui W, Yokozaki H, Shimamoto F, et al. (1999) Pathol Int 49:763–774
CDC25A overexpression	Northern	38%	D	Kudo Y, Yasui W, Ue T, et al. (1997) Jpn J Cancer Res 88:947–952
CDC25B overexpression	Northern	70%	D/P	Kudo Y, Yasui W, Ue T, et al. (1997) Jpn J Cancer Res 88:947–952
p21 reduced expression	Northern	53%	D	Akama Y, Yasui W, Kuniyasu H, et al. (1996) Mol Cell Differ 4:187–198
p21 reduced expression	IHC	46%	D	Yasui W, Akama Y, Kuniyasu H, et al. (1996) J Pathol 180:122–128
p27 reduced expression	IHC	56%	D/P	Yasui W, Kudo Y, Semba S, et al. (1997) Jpn J Cancer Res 88:625–629
E2F-1 overexpression	Northern	40%	D	Suzuki T, Yasui W, Yokozaki H, et al. (1999) Int J Cancer 81:535–538
E2F-3 reduced expression	Northern	70%	D	Suzuki T, Yasui W, Yokozaki H, et al. (1999) Int J Cancer 81:535–538
Chk1 overexpression	Western	71%	D	Shigeishi H, Yokozaki H, Oue N, et al. (2002) Int J Cancer 99:58–62
Chk2 overexpression	Western	78%	D	Shigeishi H, Yokozaki H, Oue N, et al. (2002) Int J Cancer 99:58–62

[a] Kinase, kinase assay; Southern, Southern blotting; Northern, Northern blotting; IHC, immunohistochemistry; Western, Western blotting
[b] Participation in tumor development (D) or progression (P)

expression of cyclin E tends to correlate with tumor invasion and advanced stage. The overexpression of CDC25B is found in 70% of gastric carcinoma that is associated with invasion and metastasis. On the other hand, reduction in the expression of $p27^{Kip1}$ is associated with both development and progression of gastric carcinoma. An important downsteam target of cyclins/CDKs at G_1/S transition is a family of transcription factor E2F. E2F-1 is overexpressed in 40% of gastric carcinoma and 70% of gastric carcinomas show reduced expression of E2F-3, suggesting that E2F family members may have a distinct role in stomach carcinogenesis. Chk1 and Chk2 are DNA damage-activated kinases involved in the G_2/M checkpoint. Both Chk1 and Chk2 are overex-

pressed in more than 70% of gastric carcinoma. The overexpression is associated with *p53* mutations. Therefore, Chk1 and Chk2 may play a role in checkpoint function in gastric carcinoma harboring *p53* mutation when their functions are preserved to prevent cell-cycle progression.

Angiogenic Factors

Angiogenesis, which is a prerequisite for tumor growth and metastasis, depends on the production of angiogenic factors by host and tumor cells (Fig. 2). Increased vascularity enhances the growth of primary neoplasms and provides an avenue for hematogenous metastasis. In gastric carcinoma, increasing microvessel counts correlate with lymph node metastasis, hepatic metastasis, and poor prognosis. Several growth factors have been identified that regulate angiogenesis in gastric carcinoma [4]. Gastric carcinoma cells produce various angiogenic factors, including vascular endothelial growth factor (VEGF), interleukin (IL)-8, basic fibroblast growth factor (bFGF), and platelet-derived endothelial cell growth factor (PD-ECGF) [4,16–18]. Takahashi et al. [16] have found that the angiogenic phenotype differs between the well-differentiated type and poorly differentiated type of gastric carcinoma. Well-differentiated-type tumors, but not the poorly differentiated type, highly express VEGF, whose levels significantly correlate with vessel counts. bFGF expression was higher in the poorly differentiated type, especially scirrhous-type carcinoma. A majority of gastric carcinomas express IL-8/receptor systems, and the expression levels of IL-8 directly correlate with tumor vascularity [17]. Gastric carcinoma cells transfected with the IL-8 gene produce rapidly growing and highly vascular neoplasms at the orthotopic site (gastric wall) in nude mice [19]. Furthermore, IL-8 increases the expression of EGFR, VEGF, and IL-8 itself by the tumor cells themselves [20].

The microenvironment may influence the angiogenic phenotype of gastric carcinoma. In our in vitro study, *H. pylori* infection, a candidate promoter for gastric carcinoma, increased expression of mRNA encoding IL-8, VEGF, and angiogenin by tumor cells [21]. In addition to the neoplastic cells, various interstitial cells in the tumor microenvironment may be involved in angiogenesis. Macrophage infiltration into gastric carcinoma correlates significantly with tumor vascularity and monocyte chemoattractant protein (MCP)-1 expression by tumor cells. Because the activated macrophage is a producer for VEGF, IL-8, and PD-ECGF, MCP-1 expressed by gastric carcinoma cells plays a role in angiogenesis via macrophage recruitment and activation.

Molecular Bases of Gastric and Intestinal Phenotype Gastric Carcinoma

Well-differentiated gastric carcinoma is classified into those with gastric and intestinal phenotypes by mucin histochemistry and immunohistochemistry [22]. Gastric carcinoma cells can be differentiated into a gastric epithelial cell (G) type, resembling pyloric glands and foveolar epithelia, and an intestinal epithelial cell (I) type, such as goblet and intestinal absorpitive cells, by analyzing phenotypic expression. The *p53* gene abnormalities are frequently associated with I-type carcinoma, whereas LOH of the *p73* gene, a homologue of *p53*, occurs specifically in G type with foveolar epithe-

lial phenotype [23,24]. Caudal-type homeobox (*CDX*) 1 and *CDX2* are members of the caudal-related homeobox gene family, and CDX proteins act as intestine-specific transcription factors [25]. CDX2 upregulates goblet-specific *MUC2* gene expression [26]. I-type carcinomas express CDX1 and CDX2 at high levels [25]. Liver-intestine (LI) cadherin, also known as cadherin 17 (CDH17), is overexpressed in I-type carcinoma that is correlated with tumor invasion and metastasis [27–29]. It has been shown that CDX2 binds to the promoter of *CDH17* and upregulates gene expression [30]. On the other hand, the expression of SOX2, a member of transcription factor family containing an *Sry*-like high-mobility group box, is well preserved in G-type carcinoma and down-regulated in I-type carcinoma [22]. MSI associated with *hMLH1* hypermethylation is frequent in G-type carcinoma [23]. Details of the molecular bases of gastric carcinoma with foveolar epithelial phenotype are described in chapter by Yokozaki et al. (this volume).

Epigenetic Alterations of Tumor-Related Genes

DNA Methylation

Many lines of evidence indicate that DNA methylation is important in differential control of gene expression. The abnormal methylation of CpG islands associated with tumor suppressor genes can lead to transcriptional silencing, inactivating the gene and participating in tumorigenesis. In gastric carcinoma, aberrant methylation is involved in the inactivation of various important genes such as $p16^{MTS1/INK4A}$, *CDH1* (E-cadherin), *hMLH1*, *RAR-beta*, *RUNX3*, *MGMT* (O^6-methylguanine methyltransferase), *TSP1* (thrombospondin-1), *HLTF* (helicase-like transcription factor), *RIZ1* (retinoblastoma protein-interacting zinc finger gene-1), and *CHFR* [4,31–36]. The incidence of DNA hypermethylation and inactivation of these genes in gastric carcinoma ranges from 10% to 70%. The expression is restored by treatment of 5-aza-2′-deoxyxytidine (5-aza-dC), a DNA methyltransferase inhibitor. Because these genes have respective functions, the inactivation participates in stomach carcinogenesis through abnormalities in cell-cycle regulation, cell adhesion property, signal transduction, gene regulation, DNA repair, and so on. Carcinomas frequently have the CpG island methylator phenotype (CIMP) [37]. Gastric carcinomas showing methylation at more than three of the five loci of *methylated in tumors* (MINT) were designated as CIMP positive. Significant association is found between the CIMP-positive and promoter hypermethylation of *hMLH1*, *p16*, *CDH1*, and *RAR-beta*. By a genome scanning technique, methylation-sensitive representational difference analysis, Kaneda et al. [38] found that nine CpG islands (CGIs) in the 5′-regions of nine genes, *LOX*, *HRASLS*, *bA305P22.2.3*, *FLNc* (gamma-filamin/ABPL), *HAND1*, a homologue of *RIKEN 2210016F16*, *FLJ32130*, *PGAR* (*HFARP/ANGPTL4/ARP4*), and thrombomodulin, were methylated in gastric carcinoma cell lines but unmethylated in the normal samples. These genes may include important genes in gastric carcinoma development and would be useful to identify a distinct subset of gastric carcinomas.

Alterations in DNA methylation patterns sometimes differ depending on histological type of gastric carcinoma [39,40]. Hypermethylation of *hMLH1* is frequent in pap-

illary subtype (foveolar phenotype) of well-differentiated adenocarcinomas [23]. On the other hand, CpG island methylation of *CDH-1* and reduced E-cadherin expression is commonly observed in poorly differentiated adenocarcinoma of nonsolid (scirrhous) type [39]. Methylation of *CDH1* promoter is known as the second genetic hit in hereditary scirrhous gastric carcinoma. Furthermore, CIMP and *p16* methylation are frequent in well-differentiated type or poorly differentiated solid type, whereas *RAR-beta* methylation is common in the poorly differentiated nonsolid type [40].

In addition to tumor-specific DNA methylation, some gene promoters become hypermethylated in nonneoplastic condition during aging. Alternatively, the incidence of promoter hypermethylation of *hMLH1* and *p16* is more frequent in nonneoplastic gastric mucosa of gastric carcinoma patients than in those of noncancer individuals. Although hypermethylation of *hMLH1*, *p16*, *TSP1*, and *TIMP-3* sometimes occurs in intestinal metaplasia and adenomas, the number of methylated genes increases from normal mucosa to intestinal metaplasia to adenoma to carcinoma [41]. These observations indicate that DNA methylation occurs early and accumulates along the multistep stomach carcinogenesis.

Although DNA methyltransferase and demethylase are enzymes potentially affecting promoter methylation status, tumor-specific hypermethylation is not fully understood and does not simply depend on the expression levels of promethylating (DNMT1, DNMT3A, DNMT3B) and antimethylating (MBD2) enzymes. It has been shown that DNMT1 and DNMT3B cooperate to silence genes and that DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancers [42,43].

Histone Modification and Chromatin Remodeling

Histone acetylation and chromatin remodeling linked with CpG island methylation play a major role in the epigenetic regulation of gene expression [44]. Acetylation of histones through an imbalance of histone acetyltransferases and deacetylases disrupts nucleosome structure, which leads to DNA relaxation and a subsequent increase in accessibility for transcription factors. There is a tight association between histone acetylation and DNA methylation. Histone deacetylase-1 (HDAC1) can form a complex with both methyl-CpG-binding proteins (MeCP) and DNMT1 to silence the gene expression. In contrast, methylation of histone tails is alternately linked to activation and repression, depending on the residue methylated [45]. The expression of acetylated histone H4 is reduced in 70% of gastric carcinomas, 40% of gastric adenomas, and some of the intestinal metaplasia adjacent to carcinoma, suggesting that a low level of global histone acetylation may occur even in precancerous cells [5]. Furthermore, reduced histone acetylation is significantly associated with depth of tumor invasion and nodal metastasis of gastric carcinoma. Hypoacetylation of histones H3 and H4 in the $p21^{WAF1/Cip1}$ promoter region is observed in more than 50% of gastric cancer tissues by chromatin immunoprecipitation (ChIP). Hypoacetylation of histone H3 in the promoter is associated with reduced expression of p21 regardless of *p53* gene status. A HDAC inhibitor, trichostatin A (TSA), induces growth arrest and apoptosis and suppresses invasion of gastric carcinoma cells [5]. TSA increases the expression of p21, CBP, Bak, and cyclin E, while it reduces the

expression of E2F-1, E2F-4, HDAC-1, and the phosphorylated form of Rb protein [5]. TSA also induces the expression of many suppressor genes of invasion and metastasis including TIMPs and nm23H1/H2. These findings suggest that histone deacethylation may participate not only in tumorigenesis but also in invasion and metastasis through modifying a variety of gene expression. Therefore, histone acetylation should be a promising target for cancer therapy, especially against invasive and metastatic disease.

Histone hypoacetylation and DNA hypermethylation occur concordantly in transcriptional regulation of several genes. For instance, HLTF is a homologue to SWI/SNFs, which are ATP-dependent chromatin remodeling enzymes [34]. Half of gastric cancers show DNA methylation of *HLTF* gene, whereas no gastric mucosa from healthy subjects show the methylation. Loss of HLTF expression in gastric carcinoma cells is rectified by 5-aza-dC and TSA. The acetylation levels of histones H3 and H4 in the CpG island of the HLTF are inversely associated with DNA methylation status.

Genetic Polymorphism and Gastric Carcinoma Risk

Genetic polymorphism is an important determinant for the endogenous cause of cancer. Individual variations in cancer risk are associated with genetic polymorphisms (specific variant alleles of different genes) that are present in a significant proportion of the normal population. Gonzalez et al. [46] has described an overview of genetic susceptibility and gastric carcinoma risk. Genetic susceptibility must be crucial in various processes relevant to stomach carcinogenesis, including (1) mucosal protection against *H. pylori* infection or other carcinogens; (2) the inflammatory response that conditions the maintenance, severity, and outcome of the *H. pylori* infection; (3) the functioninig of carcinogen detoxification and antioxidant protection; (4) the intrinsic variability of DNA repair processes; and (5) cell proliferation activity. Representative reports of the association between genetic polymorphism and gastric carcinoma risk are shown in Table 4. IL-1beta gene (*IL1B*) and the IL-1 receptor angagonist gene (*IL1RN*) variants *IL1B* (-31 T genotype) and *IL1RN* IVS 86bp VNTR (2/2 genotype), thought to increase IL-1beta production and to inhibit gastric acid secretion, are associated with an increased risk of chronic hypochlorhydric response to *H. pylori* infection and an increased gastric carcinoma risk. *NAT1* is responsible for *N*-acetyltransferase activity, which catalyzes acetylation and modification of aromatic and heterocyclic amine carcinogens. A significant increase of gastric carcinoma risk is associated with genotypes of NAT1 (1088 T > A, 1095 C > A). In the Japanese population, gastric cancer risk is particularly high in well-differentiated carcinoma and in heavy smokers, suggesting the involvement of NAT1 in smoking-induced stomach carcinogenesis.

As to the relation between polymorphism of tumor-related genes and cancer risk, several studies have been performed. Single nucleotide polymorphism (SNP) (A > G, *Ile* > *Val*) is present in the transmembrane domain of the *HER-2/c-erb*B2. Our case-control study has demonstrated that the *Val* genotype is significantly more frequent in gastric carcinoma patients than in controls. In patients, gastric carcinomas of advanced stage are more frequent in patients with *Val* genotype than those with *Ile*

TABLE 4. Association of genetic polymorphism with gastric carcinoma risk and progression

Gene and molecule	Site of single nucleotide polymorphism (SNP)	Role	Reference
MUC1	Coding VNTR	Risk Portuguese	Carvalho F, Seruca R, David L, et al. (1997) Glycoconj J 14:107–111
Interleukin 1 beta (*1L1B*)	Promoter –31 C/T	Risk	El-Omar EM, Carrington M, Chow WH, et al. (2000) Nature (Lond) 404:398–402
Interleukin 1 receptor antagonist (*IL1RN*)	IVS2 86-bp VNTR	Risk	El-Omar EM, Carrington M, Chow WH, et al. (2000) Nature (Lond) 404:398–402
N-Acetyltransferase 1 [*NAT1*]	1088 T/A, 1095 C/A	Risk	Katoh T, Boissy RJ, Nagata N, et al. (2000) Int J Cancer 85:46–49
Cytochrome P450 2E1 (*CYP2E1*)	–1053 C/T	Risk Brazilians	Nishimoto IN, Hanaoka T, Sugimura H, et al. (2000) Cancer Epidemiol Biomark Prev 9:675–680
Glutathione *S*-transferase P1 (*GSTP1*)	Coding Ile105Val	Risk?	Katoh T, Kaneko S, Takasawa S, et al. (1999) Pharmacogenetics 9:165–169
Methylenetetrahydrofolate reductase (*MTHFR*)	Coding Ala677Val	Risk Chinese	Shen H, Xu Y, Zheng Y, et al. (2001) Int J Cancer 95:332–336
HER-2/c-*erb*B2	Coding Ile 665 Val	Risk	Kuraoka K, Oue M, Matsumura S, et al. (2003) Int J Cancer 107:593–596
MMP-1	Promoter –1607 G/GG	Histology	Matsumura S, Oue N, Kitadai Y, et al. (2004) J Cancer Res lin Oncol 130:259–265

genotype, suggesting that this SNP could modulate gastric cancer risk and serve as a predictor of risk for a malignant phenotype. Matrix metalloproteinase-1 (MMP-1) plays a key role in cancer invasion and metastasis. There is 1G/2G SNP in the promoter region of the *MMP-1* affecting the transcriptional activity. Although no difference has been found in the frequency of 1G/2G genotype between gastric carcinoma patients and controls, a significant association is detected with histological differentiation. The 2G genotype is more frequent in poorly differentiated gastric carcinoma than in well-differentiated tumors. Controversial observations have been reported in the association between *CDH1* (E-cadherin) promoter (−160 C > A) polymorphism and the risk of gastric carcinoma. One report indicates that individuals with A/A genotype have a decreased risk of gastric carcinoma [47], whereas another shows no difference in genotype frequencies between gastric carcinoma cases and controls [48]. The important limitations in case-control studies that preclude definitive conclusions are lack of appropriate control, low number of cases analyzed, and lack of concomitant analysis with exposure to relevant cofactors such as *H. pylori* infection and smoking. Proper association studies between genetic polymorphism and cancer risk and genotype information in individuals must be important because those factors directly connect with personalized cancer prevention. Furthermore, genetic polymorphisms have been associated with therapeutic efficacy and toxicity of anticancer drugs [49]. For instance, polymorphism of VNTR in the promoter region of thymidylate synthase influences response to 5-fluorouracil. Polymorphism (difference in number of TA repeats) in the promoter region of the UDP-glucuronosyltransferase 1A1 gene affects severity of toxicity during irinotecan (CPT-11) therapy.

Novel Genetic Markers Identified by Gene Expression Profile

Microarray Study

Cancer is accompanied by multiple genetic and epigenetic alterations, including mutation, gene amplification, LOH, gene silencing by DNA methylation, and loss of imprinting, all of which modify gene expression profiles. Therefore, genome-wide study of gene expression is greatly important to uncover the precise mechanism of development and progression of cancer. Microarray technology provides high-throughput analysis of gene expression profiles by means of small-array slides. cDNA microarray, array slides spotted with cDNAs, is commonly used to detect differences between tumor and normal cells among various histologies and clinical outcomes, for example. The use of laser capture microdissection and T7-based RNA amplification helps to study gene expression profile in a small amount of sample with minimal contamination of other components than those of interest.

Several microarray studies have been performed on gastric carcinoma. El-Rifai et al. [50] examined the gene expression profile of gastric carcinoma using cDNA microarray with 1200 genes and found that S100A4, CDK4, MMP14, and beta catenin are the most upregulated in gastric carcinoma. Hippo et al. [28] studied the expression profile of 6800 genes and identified 162 that were highly expressed in gastric carcinoma tissues; these included genes related to cell cycle, growth factor, cell motility,

cell adhesion, and matrix remodeling. They also found several genes associated with metastasis, including Oct-2, a POU domain transcription factor, or intestinal histology, including CDH17 and LI-cadherin. Hasegawa et al. [51] performed genome-wide analysis of gene expression in well-differentiated gastric cancer using a cDNA microarray representing 23040 genes and reported that 61 genes and 63 genes were commonly up-regulated and downregulated, respectively, in gastric carcinoma. Altered expression of 12 genes including *DDOST*, *GNS*, *NEDD8*, *LOC51096*, and *AIM2* was found to be associated with lymph node metastasis. Hasegawa et al. developed a "predictive score" based on the expression profiles of these five genes that could distinguish cancers with metastasis from those without metastasis. A similar approach has been carried out by Inoue et al. [52] to develop a prognostic scoring system using cDNA microarray. They selected 78 genes that were differentially expressed between aggressive and nonaggressive groups with respect to conventional pathological parameters and determined a coefficient for each gene. The prognostic score, calculated by summing up the value for each gene, could predict stage of disease and the patient's prognosis. Those strategies can be applicable to identify genes associated with sensitivity of cancer to anticancer drugs [53]. These observations indicate that the gene expression profile and a scoring system based on microarray analysis have great potential for dissecting the character of gene expression in individual cancers and predicting biological behavior and chemosensitivity.

Serial Analysis of Gene Expression (SAGE)

Besides microarray technique, serial analysis of gene expression (SAGE) is a powerful technique to allow global analysis of gene expression in a quantitative manner without prior knowledge of the sequence of the genes [54]. SAGE is based on the following principles. A short nucleotide sequence tag (about 10 base pairs) is sufficient to uniquely identify a transcript, provided it is isolated from a defined position within the transcript. Concentration of short sequence tags allows the efficient analysis of transcripts in a serial manner by the sequencing of multiple tags within a single clone. Because the SAGE tag numbers directly reflect the abundance of the mRNA, SAGE data are highly accurate and quantitative, and completion of the human genome sequence has facilitated the mapping of specific genes to individual tags. Up to now, four SAGE studies of gastric carcinoma, including ours, have been reported that identified several upregulated and downregulated genes [55–58]. Our SAGE study on five samples of gastric carcinoma of different stages and histology from four patients generated a total of 137706 tags including 38903 unique tags [58]. Our SAGE libraries are the largest gastric carcinoma libraries in the world, and sequence data from our SAGE libraries are publicly available at SAGEmap (GEO accession number GSE 545, SAGE Hiroshima gastric cancer tissue) (*http://www.ncbi.nlm.nih.gov/SAGE/*).

Comparison between SAGE tags from gastric carcinoma and those from normal gastric epithelia identifies upregulated and downregulated genes that may participate in stomach carcinogenesis (Table 5) [29,58]. If SAGE libraries are compared between early cancer and advanced cancer or between primary tumor and metastatic tumor, candidate genes involved in invasion and metastasis can be identified. The upregulated genes in gastric carcinoma include *APOC1*, *NDUF2*, *TEBP*, *COL1A1*, and so on, in addition to *TFF3* and *S100A4*, which are known to be upregulated in gastric carci-

noma [58]. Quantitative real-time reverse transcription-PCR (RT-PCR) confirmed that *APOC1*, *CEACAM6*, and *YF13H12* are frequently overexpressed. The downregulated gene cluster includes *LIPF* (gastric lipase), *CHIA*, *ATP4B*, *MBD3*, and many unknown genes. By comparing gene expression profiles between gastric carcinomas at early and advanced stages, several differentially expressed genes by tumor stage were also identified, including *FUS*, *CDH17*, *COL1A1*, and *COL1A2*, that should be novel genetic markers for high-grade malignancy. *FUS* is a tumor-associated fusion gene, especially in myxoid liposarcoma, and its possible role is supposed to be to regulate transcription and maintain chromosomal stability [59]. Regarding genes involved in metastasis, the 20 most upregulated tags and corresponding genes in the

TABLE 5. Upregulated and downregulated tags and genes in gastric carcinoma obtained by serial analysis of gene expression (SAGE)

Commonly upregulated and downregulated tags and genes in gastric carcinoma in comparison with normal gastric epithelia	
Upregulated	APOC1, S100A4, NDUF2, TEBP, COL1A2, SUFU, SYAP1, KIAA0930, KIAA1694, TFF3, CEACAM6, FLJ20249, FLJ2167, EIF4A1, COLPH2, G3BP, YF13H12, KRT7, SH3BP2, COL1A1, LOC284371
Downregulated	CAGCGCTTCT (no match), CACCTCCCCA (no match), AGCCTCCCCA (no match), ACCCTCCCCA (no match), LIPF, AACCTCCCCC (no match), CHIA, TAGTGCTTCT (no match), TACAAGGTCC (no match), GTGGTCAGCT (no match), ATP4B, FLJ20410, MBD3, CAGTGCTTTT (no match), Hs.199360, Hs.353061
The 20 most upregulated and downregulated tags and genes in advanced carcinoma in comparison with early carcinoma[a]	
Upregulated	TCCCCGTAAA (no match), TCCCGTACAT (no match), CDH17, FUS, PRO1073, FLJ36926, FLJ30146, PAI-RBP1, COL1A2, TCCTATTAAG (no match), COL1A1, GRAP2, HNRPL, NUTF2, ERP70, PES1, CYP2J2, DAG1, IQGAP1, IL16, FXYD3, COQ4, LOC91966, CTBP1, TTCGGTTGGT (no match), alpha4GnT, Hs.290723, AKT3, CCT3, HMG20A
Downregulated	Hs.216636, LOC116228, SH3MD2, NAB1, TTCCCCCAAA (no match), DDX5, VMP1, LOC51123, LZK1, CGCAGATCAG (no match), IFRD2, Hs.284464, RPS4Y, RPS4Y2, UAP1, Hs.180804, CATTAAATTA (no match), IKBKAP, ARPC3, NAGA, UBE3A, TRAG3, PNN, CTAATTCTTT (no match), TCCATCGTCC (no match)
The 20 most upregulated and downregulated tags and genes in metastatic tumor in comparison with primary tumor of gastric carcinoma[a]	
Upregulated	SCAND1, RGS5, S100A11, RNPC2, APOE, FLJ10815, RNASE1, H3F3B, P24B, LOC151103, CLDN3, MRPL14, PRex1, TCCCCTATTA (no match), Hs.105379, ATP5G1, NPD007, MGC3180, WDR11, ARPC1B, ABTB2, DNAJB1, HMGN2, KIAA1393, RAP1B, FLJ12150, STUB1
Downregulated	ERdj5, RPL27A, DHRS3, E2IG5, USP7, CTSL, KRTHB1, KRTHB3, TGCACTACCC (no match), ALG12, S100A9, CTAGCTTTTA (no match), ELOVL5, LOC375463, GGGGGAGTTT (no match), ACTGCCCTCA (no match), SPC18, CTNND1, CYP20A1, FLJ11151, RPS17, ZYX, RPS16, GCTTTCTCAC (no match), BCL2L2

Symbol of gene is described; UniGene ID is described if symbol is not present
No match, tag sequence is not matched to known gene
[a]Because some genes share the same SAGE tag, gene numbers are more than 20

metastatic tumor of gastric carcinoma included *SCAN D1*, *RGS5*, *S100A11*, *RNPC2*, and *APOE* [58]. *APOE* (apolipoprotein E) expression is associated with T grade, N grade, and advanced stage.

SAGE is also useful to isolate novel biomarkers of gastric carcinoma. The ideal biomarker should be overexpressed in a majority of gastric carcinoma and expressed on the cell surface or secreted to facilitate its detection. Moreover, if the function of the gene product is involved in the neoplastic process, the gene is not just a biomarker but can be a therapeutic target. One example is *REGIV* (regenerating gene type IV), which is identified by comparing the expressed tags of poorly differentiated nonsolid type (scirrhous-type) gastric carcinoma with those of normal gastric epithelia [58,60]. About half of gastric carcinomas overexpress *REGIV* mRNA regardless of tumor stage and histological differentiation. In vitro studies using *RegIV*-transfected cells revealed that RegIV is secreted by carcinoma cells and that RegIV inhibits apoptosis, suggesting that RegIV may serve as a novel biomarker and therapeutic target for gastric carcinoma. Other examples include *GW112* and *MIA*, both of which encode secreting proteins [61,62]. GW112 demonstrates strong antiapoptotic effects in cancer cells treated with stress exposures and forced expression of GW112 leads to more rapid tumor formation, indicating that GW112 plays an important role in tumor cell survival and growth and should be a good therapeutic target [61].

Clinical Implication of Global Gene Expression Analysis

A strategy to clinical applications of global analysis of gene expression such as diagnostics, treatment, and prevention is shown in Fig. 3. According to gene expression profiles among gastric carcinomas or with those in normal gastric tissue obtained by microarray study or SAGE, specifically upregulated or downregulated genes are identified. The expression of these genes is confirmed in a large number of cases by real-time RT-PCR and immunohistochemistry if antibodies are available. With the specific genes identified by SAGE, known genes participating in the development and progression of gastric carcinoma and known genetic markers for chemosensitivity, a custom-made cDNA microarray is prepared. If the specific gene encodes secretory protein, this may be detected in the blood and should be a novel biomarker of gastric carcinoma. For such molecules, DNA/RNA aptamer or antibody is produced to establish a measuring system such as enzyme-limited immunosorbent assay (ELISA) in blood sample. These methods can be applied for clinical diagnosis and cancer detection. Polymorphism of genes, highly altered in their expression in gastric carcinoma, may be candidates of novel risk factors, and this information will be used for cancer prevention. By functional analysis, the molecular mechanism of stomach carcinogenesis can be understood in more detail and the possibility whether the genes are novel therapeutic targets can be revealed. Combination of these testings not only can attain cancer detection but also can clarify the character of an individual tumor and person, which is directly connected with personalized medicine and cancer prevention.

Conclusion

In the course of multistep carcinogenesis of the stomach, various alterations of oncogenes, tumor suppressor genes, DNA repair genes, growth factors/receptors, cell-cycle

regulators, and cell adhesion molecules are accumulated. Some of these changes occur commonly in both well-differentiated and poorly differentiated types and some differ depending on the histological types. Among various epigenetic alterations, modified gene expression through DNA methylation and chromatin remodeling by histone modification are the most important events. Genetic polymorphism is a crucial endogenous cause and fundamental factor of cancer risk. Using genomic science including novel techniques for global analysis of gene expression and bioinformatics, the individual character of each person and cancer can be dissected precisely, which is directly connected to personalized medicine and cancer prevention. Understanding of the diversity of gastric cancer must be critical in the era of genomic medicine at the clinical setting.

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