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Special article

Molecular-pathological prognostic factors of gastric cancer: a review

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Abstract

Invasion and metastasis are critical determinants of cancer morbidity. Genes and molecules participating in these steps must be regarded as potential prognostic factors. Growth factors and their receptors, cell-cycle regulators, cell-adhesion molecules and matrix-degrading enzymes are those to be used as prognostic factors, including epidermal growth factor (EGF), EGF receptor, K-*sam*, *HER-2*, interleukin (IL)-8, vascular endothelial growth factor (VEGF), cyclin E, p27, E-cadherin, CD44v6, matrix metalloproteinase-1 (MMP-1), and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1). Alterations in epigenetics, such as aberrant DNA methylation and histone modification that are, in part, associated with the tumor progression of gastric cancer, can be candidate prognostic factors. The number of methylated genes may serve as a marker of tumor progression. Genetic polymorphism not only affects cancer susceptibility but also influences malignant phenotype; examples include single-nucleotide polymorphism in the *HER-2* and *MMP-9* genes. Comprehensive gene expression analyses are useful to search for novel genes related to invasion and metastasis and potential prognostic factors. Serial analysis of gene expression (SAGE) has identified several these genes, such as *CDH17*, *APOE*, *FUS*, *COL1A1*, *COL1A2*, *GW112*, and *MIA*. Overexpression of MIA is found to be associated with poor prognosis. Microarray analysis has great potential for identifying the characteristics of individual cancers, from the view point of gene expression profiles. A combination of these examinations can not only foretell a patient's prognosis but can also give information directly connected with personalized cancer medicine and prevention.

Key words Prognostic factor · Gastric cancer · Epigenetics · Genetic polymorphism · Serial analysis of gene expression

Introduction

Advances in diagnosis and treatment have offered excellent long-term survival for early gastric cancer; however, the prognosis of advanced cancer still remains poor. Cancer morbidity results in large part from metastases, and a majority of patients with advanced cancer die due to complications by metastases, not by the primary tumor. Integrated research in molecular pathology over the past 15 years has uncovered the molecular mechanism of invasion and metastasis in gastric cancer [1–5]. To produce a metastasis, tumor cells must complete a multistep progression through a series of sequential and selective events [6]. The metastatic process consists of tumor cell detachment, local invasion, motility, angiogenesis, vessel invasion, survival in the circulation, adhesion to endothelial cells, extravasation, and regrowth in different organs (Fig. 1). In each step, causative molecules have been identified; these include cell-adhesion molecules, various growth factors, matrix degradation enzymes, and motility factors, and most of these can be regarded as prognostic factors. Recent advances in genomic science have enabled us to uncover the detailed molecular mechanism of stomach carcinogenesis and its progression. A better knowledge of the molecular bases will lead to new paradigms and possible improvements in diagnostics and therapeutics. Analyses of gene expression profiles and genetic polymorphisms are approaches to identify novel prognostic factors. This review describes changes in genes and molecules to be used as prognostic factors of gastric cancer, and their application in the clinical setting.

Classical prognostic factors

Genes and molecules participating in proliferation, invasion, and metastasis, such as growth factors and their receptors, cell-cycle regulators, cell-adhesion mol-

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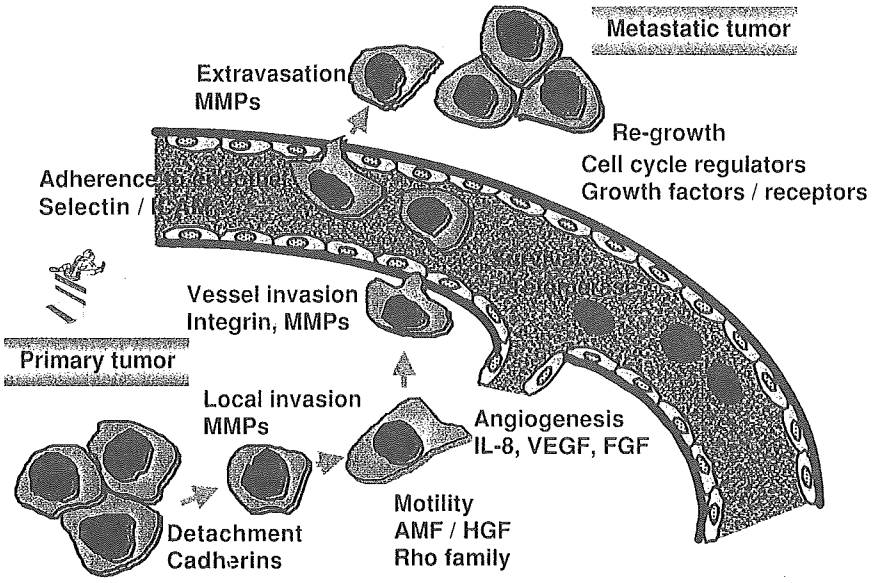

Fig. 1. Molecular mechanism of hematogenous metastasis. *MMPs*, matrix metalloproteinases; *IL*, interleukin; *VEGF*, vascular endothelial growth factor; *FGF*, fibroblast growth factor; *AMF*, autocrine motility factor; *HGF*, hepatocyte growth factor; *ICAM*, intercellular adhesion molecule

Table 1. Molecular and genetic markers related to invasion, metastasis, and prognosis

Category	Molecular and genetic markers
Growth factor	EGF, TGF-alpha, EGF receptor, c-met, K-sam, HER-2, IL-8, VEGF
Cell-cycle regulator	Cyclin E, p27, p53, RB, CDC25B
Telomere	POT1
Cell adhesion molecules	E-cadherin, dysadherin, CD44v6, CD44v9
Matrix metalloproteinase	MMP-1, MMP-2, MT1-MMP, TIMP-1

ecules, and matrix-degrading enzymes are good prognostic factors (Table 1).

Growth factors, cytokines, and angiogenic factors

Gastric cancer cells express a variety of growth factors and their receptors to make autocrine and paracrine loops [1,2,4]. These factors induce not only cell growth but also extracellular matrix degradation and angiogenesis for tumor invasion and proliferation. The simultaneous expression of epidermal growth factor (EGF)/transforming growth factor (TGF)-alpha and EGF receptor correlates with deep invasion, advanced stage, and poor prognosis. The amplification of the c-*met* encoding receptor for hepatocyte growth factor is frequently associated with poor prognosis of gastric cancer, especially of scirrhous type. The amplification and overexpression of the K-*sam* and *HER-2/c-erbB2* genes may be prognostic factors for well-differentiated type and poorly differentiated or scirrhous type, respectively [2,7]. Angiogenesis is a prerequisite for tumor growth and metastasis that depends on the production of angiogenic factors by host and tumor cells. Neovascularization enhances the growth of primary tumors and provides an avenue for hematogenous metastasis. Gastric cancer 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) [8–10]. Because increasing vascularity correlates with lymph-node metastasis, hepatic metastasis, and poor prognosis, all of these may be candidate prognostic factors of gastric cancer. In fact, the prognosis in patients with tumors displaying high IL-8 and VEGF expression levels is significantly poorer than that in patients whose tumors with low expression levels [11].

Cell-cycle regulators

Cell-cycle checkpoints are regulatory pathways that control cell-cycle transitions, DNA replication, and chromosome segregation. Abnormalities in cell-cycle regulators are involved in stomach carcinogenesis through genomic instability and unbridled cell proliferation [2,4,12]. The cyclin E gene is amplified in 15%–

20% of gastric cancers, and the overexpression of cyclin E correlates with the aggressiveness of the cancer. Reduction in the expression of $p27^{Kip1}$, a cyclin-dependent kinase (CDK) inhibitor, is frequently associated with advanced gastric cancers, and the reduced expression of $p27^{Kip1}$ also significantly correlates with deep invasion and lymph-node metastasis. It has been shown that reduced p27 expression is a negative prognostic factor for patients with a cyclin E-positive-tumor [13]. Aberrant expression (reduced or overexpression) of the *p16* gene is frequently found in gastric cancers, but does not correlate with patients' prognosis [14]. An important regulator at the G1/S checkpoint is retinoblastoma (RB) protein. RB expression is lower in lymph-node metastasis than in the corresponding primary tumors [15]. Univariate and multivariate survival analyses have revealed that reduced expression of RB is associated with worse overall survival. The product of the tumor suppressor gene, *p53*, is multifunctional and participates in cell-cycle regulation partly through p21 induction. Although nearly 200 articles concerning *p53* abnormality in gastric cancer in relation to patients' prognoses have been published, the prognostic impact remains controversial. Recent reports indicate that abnormal expression of *p53* significantly affects cumulative survival and that *p53* status may also influence response to chemotherapy [16,17]. The overexpression of checkpoint kinase 1 (Chk1) and Chk2, DNA damage-activated kinases involved at the G2/M checkpoint, is associated with *p53* mutations, but has no prognostic impact. The overexpression of CDC25B is found in 70% of gastric cancers, and is associated with invasion and metastasis.

Genetic instability

Dysfunction of the DNA mismatch repair system is responsible for microsatellite instability (MSI). MSI causes accumulation of genetic alterations, and participates in the pathogenesis of sporadic gastric carcinomas, in addition to hereditary nonpolyposis colorectal cancer (HNPCC) [4]. The frequency of MSI is estimated to be around 30% in gastric cancers, with an especially high frequency in well-differentiated gastric carcinoma of foveolar phenotype with papillary morphology. Many reports have demonstrated the relation between MSI and cancer multiplicity [18–20]. All show that the frequency of MSI is significantly higher in patients with multiple primary cancers. Therefore, the detection of MSI may serve as a good indicator for the assessment of a second cancer risk in the same patient. There have been many studies examining the relation between MSI and the prognosis of patients' with gastric cancer [21–24]. Most studies have shown that MSI is associated with less aggressive behavior and favorable survival, while some indicated no prognostic impact.

Telomeres and telomerase

The maintenance of telomeres by telomerase activation induces cellular immortalization and participates in carcinogenesis [25]. Strong telomerase activity associated with human telomerase reverse transcriptase (*hTERT*) expression is present in a majority of gastric carcinomas, regardless of tumor staging [4,15,26]. Protection of telomeres 1 (POT1), a telomere end-binding protein, is proposed not only to cap telomeres but also to recruit telomerase to the ends of chromosomes [27]. POT1 expression levels are significantly higher in gastric cancer of advanced stage, and POT1 downregulation is frequently observed in gastric cancers of early stage, suggesting that POT1 may be a marker of high-grade malignancy [27].

Cell-adhesion molecules

Cell-adhesion molecules may function as tumor suppressors. E-cadherin plays a major role in epithelial tissues to regulate morphogenesis and inhibit cell infiltration. Multivariate analyses have revealed that reduced E-cadherin expression is an independent prognostic factor [28]. Dysadherin, a cancer-associated cell-membrane glycoprotein, downregulates E-cadherin expression and promotes metastasis [28]. Patients with both dysadherin positivity and reduced E-cadherin have the worst prognosis, although dysadherin is not an independent prognostic factor [28]. Soluble fragment of E-cadherin is known to be increased in the sera of cancer patients [29]. Serum soluble E-cadherin is a valid prognostic marker for gastric cancer, and a high concentration predicts palliative/conservative treatment and extensive tumor invasion [29]. CD44 is an important cell-adhesion molecule, and its variants, generated by alternative splicing, modulate cell-to-cell interaction, movement, and finally metastatic potential. There is a significant survival advantage in patients with low expression of CD44 sharing variant exon 6 (CD44v6) compared with those with high expression [30]. Furthermore, The serum level of soluble CD44v6 is a prognostic indicator in patients with poorly differentiated type gastric cancer [31]. The expression of CD44v9 is associated not only with tumor invasion, metastasis, and advanced stage but also with the tumor-recurrence mortality of gastric cancer [4,32].

Matrix metalloproteinases (MMPs)

A balance of activities between matrix-degrading enzymes and their inhibitors is important in determining tumor invasion and metastasis. Among various MMPs, The expression of MMP-7, also known as matrilysin, is correlated with vessel invasion and both lymphatic and hematogenous metastases [33], while the prognosis of

patients with MMP-1-positive tumors is significantly worse than that of patients with MMP-1-negative tumors [34]. Membrane-type 1 (MT-1) MMP is an activator of MMP-2. MT1-MMP expression is an independent factor influencing both tumor invasion and metastasis. Although MT1-MMP is not an independent prognostic factor, patients with tumors having a high tumor/normal (T/N) ratio of MT1-MMP show a significantly poorer prognosis than those with a low ratio [35]. Tissue inhibitors of MMP (TIMPs) inhibit tumor invasion through the inactivation of MMPs. In a multivariate analysis, the T/N ratio of TIMP-1 was shown to be an independent factor influencing tumor invasion and the second most important factor in determining the prognosis of the patients [36].

Epigenetic alterations as prognostic factors

Among the various epigenetic alterations that lead to modified gene expression, the most important are believed to be DNA methylation and chromatin remodeling by histone modification [5]. Some aberrant epigenetics modifications are associated with tumor progression of gastric cancer, and could be candidate prognostic factors.

Histone acetylation

Inactivation of chromatin by histone deacetylation is involved in the transcriptional repression of several tumor suppressor genes, including $p21^{WAF1/CIP1}$. 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 [37]. By using anti-acetylated histone antibody, the global acetylation status of histone can be analyzed immunohistochemically in tissue specimens of gastric cancer [5]. The level of acetylated histone H4 expression is reduced in 70% of gastric cancers in comparison with non-neoplastic mucosa, indicating global hypoacetylation in gastric cancer. Reduced expression of acetylated histone H4 correlated well with advanced tumor stage, deep tumor invasion, and lymph-node metastasis. Thus, low levels of global histone acetylation may serve as a marker of high-grade malignancy. In fact, trichostatin A, a histone deacetylase inhibitor, induces growth arrest and apoptosis and suppresses the invasion of gastric cancer cells [5,38].

Accumulation of DNA methylation in multiple genes

The hypermethylation of CpG islands is associated with the silencing of various tumor suppressor genes and participates in tumorigenesis. These genes include $p16^{MTS1/INK4A}$, *CDH1* (E-cadherin), *hMLH1*, *RAR-beta*, *RUNX3*, *MGMT*, *TSP1*, *HLTF*, *RIZ1*, and *SOCS-1* [4,39–45]. Among these, DNA methylation of *CDH1*, *RAR-beta*, and *SOCS-1* is significantly associated with tumor invasion and metastasis. Gastric cancers frequently have the CpG island methylator phenotype (CIMP), which may be an important pathway involved in stomach carcinogenesis [46]. However, no significant association has been found between CIMP and tumor progression. We analyzed DNA methylation in 12 tumor-related genes (*hMLH1*, *MGMT*, *p16*, *CDH1*, *RAR-beta*, *HLTF*, *RIZ1*, *TM*, *FLNs*, *LOX*, *HRASLS*, *HAND1*) in gastric cancers and found that the average number of methylated genes per tumor was about five. We then divided cancers into two groups; cancers with five or more methylated genes (high-methylation group) and those with four or fewer methylated genes. The high-methylation group was found more frequently in stage III and stage IV cancers than in stages I and II. Thus, the number of methylated genes may serve as a molecular marker of tumor progression, although the prognostic implication remains to be elucidated.

Genetic polymorphism as a prognostic factor

Genetic polymorphism is an important determinant of endogenous causes of cancer. An overview of genetic susceptibility and gastric cancer risk has been described by Gonzalez et al. [47]. Representative genetic polymorphisms modifying gastric cancer risk include IL-1beta (*IL1B*), IL-1 receptor antagonist (*IL1RN*), and N-acetyltransferase (*NAT1*). Regarding the relation between genetic polymorphisms of tumor-related genes and gastric cancer, we have performed case-control and case-case studies, in about 500 subjects [48–52]. A single-nucleotide polymorphism (SNP; A > G, Ile > Val) is present in the transmembrane domain of the *HER-2/c-erbB2* gene, while there are SNPs in the promoter regions of the *EGF* (61 A/G), *E-cadherin* (-160 C/A), *MMP-1* (-1607 1G/2G), and *MMP-9* (-1562 C/T) genes. All the promoter SNPs described above are known to influence the respective gene expression. As shown in Table 2, our case-control study showed that SNPs of the *HER-2, EGF*, and *E-cadherin* genes significantly affected gastric cancer risk, while the genotypes of the *MMP-1* and *MMP-9* genes did not differ between the gastric cancer cases and the controls. Among the gastric cancer patients, the genotypes of the *HER-2*, *E-cadherin*, and *MMP-9* genes were associated with tumor invasion, metastasis, or stage grouping. As for the *MMP-1* gene, a significant association was detected only with histological differentiation. Therefore, SNPs of the *HER-2*, *E-cadherin*, and *MMP-9* genes could serve as a predictor of risk for a malignant phenotype. The prog-

Table 2. Single-nucleotide polymorphisms of five genes and relation to relation to cancer risk and progression

Gene (substitution)	Case-control	Gastric cancer cases[d]			
		T grade	N grade	Stage	Histology
HER-2/c-erbB2 (Ile655Val)	$P = 0.033$[a] OR 3.25[b] (1.08–9.76)[c]	$P = 0.026$ 2.18 (1.11–4.30)	$P = 0.001$ 2.18 (1.11–4.30)	$P < 0.001$ 3.49 (1.84–6.63)	$P = 0.068$ 1.83 (0.90–3.72)
EGF (61 A/G)	$P = 0.012$ OR 0.56 (0.35–0.89)	$P = 0.043$ 1.80 (0.98–3.30)	$P = 0.035$ 1.98 (1.01–3.89)	$P = 0.008$ 2.26 (1.21–4.22)	$P = 0.034$ 1.89 (1.04–3.45)
E-cadherin (-160 C/A)	$P = 0.003$ OR 2.68 (1.50–4.79)	$P = 0.001$ 4.95 (2.02–12.1)	$P = 0.010$ 2.86 (1.28–6.36)	$P = 0.004$ 3.41 (1.46–7.94)	$P = 0.029$ 2.31 (1.02–5.24)
MMP-1 (-1607 1G/2G)	$P = 0.571$ OR 0.83 (0.43–1.59)	$P = 0.904$ 1.02 (0.24–4.35)	$P = 0.919$ 1.14 (0.16–8.13)	$P = 0.271$ 3.04 (0.83–11.2)	$P = 0.03$ 3.56 (1.15–11.1)
MMP-9 (-1562 C/T)	$P = 0.223$ OR 0.765 (0.49–1.18)	$P = 0.03$ 2.61 (1.07–6.34)	$P = 0.23$ 1.54 (0.76–3.10)	$P = 0.02$ 2.26 (1.12–4.55)	$P = 0.20$ 1.60 (0.78–3.28)

[a] Correlation was analyzed by Fisher's exact test
[b] OR, Odds ratio. ORs were adjusted for age and sex
[c] 95% CI, 95% confidence interval
[d] Tumor grade was classified according to the criteria of the UICC TNM stage grouping. Histology was classified according to the criteria of Lauren

nostic significance must be clarified. It should be mentioned that controversial observations have been reported in regard to the association between *E-cadherin* SNP and gastric cancer [53,54].

Novel prognostic factors identified by gene expression profiles

A genome-wide study of a gene expression profile is of great advantage to uncover the precise mechanism of the development and progression of cancer and to identify novel biomarkers of malignancy that could be candidate prognostic factors.

Serial analysis of gene expression (SAGE)

SAGE is a powerful technique that allows the global analysis of gene expression in a quantitative manner, without prior knowledge of the sequence of the genes [55,56]. Among the four SAGE studies of gastric cancer reported [57–60], ours [60] has described the largest SAGE libraries of gastric cancer in the world, and sequence data are publicly available at SAGEmap (Gene Expression Omnibus [GEO] accession number GSE 545, SAGE Hiroshima gastric cancer tissue). By comparing gene expression profiles between gastric cancer and normal gastric mucosa, in combination with quantitative reverse transcription-polymerase chain reaction (RT-PCR), *COL1A1*, *CDH17*, *APOC1*, *COL1A2*, *YF13H12*, *CEACAM6*, *APOE*, *REGIV*, *S100A11*, and *FUS* were found to be overexpressed in over 40% of gastric cancers [60]. Candidate genes involved in invasion and metastasis can be identified by comparing SAGE libraries between early cancer and advanced cancer or between primary tumor and metastatic tumor. Quantitative real-time RT-PCR confirmed that the expression of *CDH17*, *APOE*, *FUS*, *COL1A1*, and *COL1A2* was associated with tumor invasion, metastasis, and stage grouping, indicating that these could be novel genetic markers for high-grade malignancy. In fact, the prognosis of CDH17-positive patients is significantly worse than that of -negative patients [56].

If a gene participates in tumor progression and is specifically expressed in cancer but not in normal tissues, the gene can be not only a cancer biomarker but also a therapeutic target, with minimal adverse effects [56]. By comparing SAGE libraries of gastric cancer with those of various normal tissues in the SAGEmap database, we identified about 60 genes that were detected in our gastric cancer libraries, but not in the libraries from 12 normal tissues, including brain, lung, heart, liver, and kidney. We then validated the expression of these genes in gastric cancers and normal human tissues by quantitative RT-PCR and found that 8 genes, including *MIA* (melanoma inhibitory activity) and *GW112*, were specifically expressed in gastric cancer (Fig. 2). MIA was first isolated as a secreted protein from malignant melanoma cell lines [61]. MIA enhances migration and invasion ability and inhibits apoptosis;

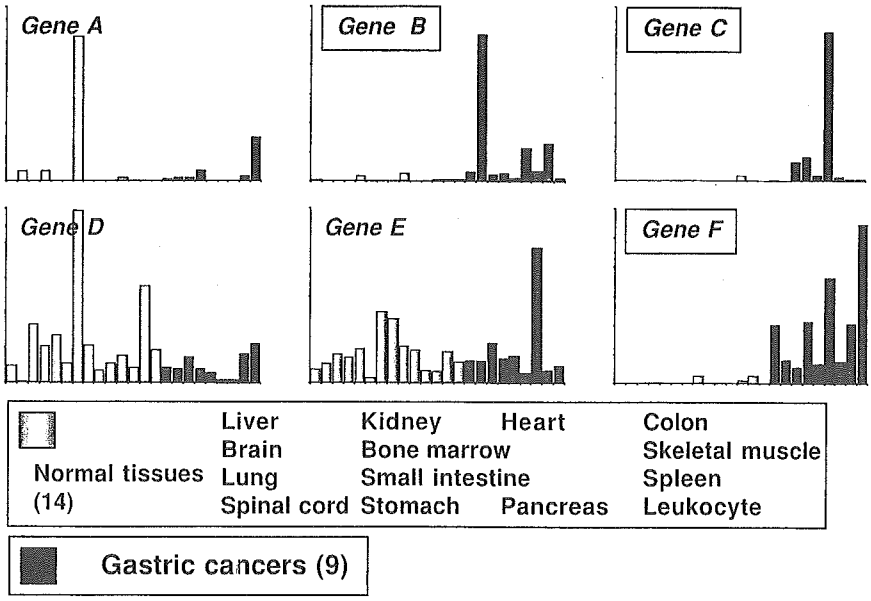

Fig. 2. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) of various normal tissues and gastric cancers. The expression of genes *B*, *C*, and *F* is confined to gastric cancers, while that of genes *A*, *D*, and *E* is detected in both normal tissues and gastric cancers at various levels

increased serum levels of MIA are correlated with the progression of melanoma [62,63]. The expression levels of *MIA* were correlated with advanced tumor stage of gastric cancer. Immunostaining of MIA demonstrated a significant association with tumor stage in gastric cancer patients, and high levels of MIA were detected in the sera of stage IV patients by enzyme-linked immunosorbent assay (ELISA). Patients with MIA-positive cancer showed poorer prognoses than those with MIA-negative cancer. This approach provides a list of candidate genes that may serve not only as prognostic factors but also as good therapeutic targets of gastric cancer.

Microarray-based molecular and genetic analysis

Several microarray studies have been performed in gastric cancer to find gene expression signatures specifically related to metastasis and prognosis. Hippo et al. [64] studied the expression profiles of 6800 genes and reported that overexpression of *RBP4*, *OCT2*, *IGF2*, *PFN2*, *KIAA1093*, *PCOLCE*, and *FN1* was associated with lymph-node metastasis. Hasegawa et al. [65] performed a genome-wide analysis of gene expression in well-differentiated gastric cancer, using a cDNA microarray representing 23040 genes, and found that the altered expression of 12 genes (*DDOST*, *GNS*, *NEDD8*, *LOC51096*, *CCT3*, *CCT5*, *PPP2R1B*, *UBQLN1*, *AIM2*, *USP9X*, and two expressed sequence tags [ESTs]) was associated with lymph-node metastasis. Inoue et al. [66] developed a prognostic scoring system using a cDNA microarray. Seventy-eight genes (including *MMP-7*, *SPARC*, *TGFB3*, *THBS2*, *PCNA*, *CEACAM6*, *FN1*, *IGFBP3*, and *CSPG2*) were differentially expressed in patients with aggressive and nonaggressive gastric cancers. The prognostic score, calculated by summing-up the value of a coefficient for each gene, can predict the stage of disease and the patient's prognosis. We have developed a custom-made oligo-DNA microarray including specific genes identified by our SAGE analysis, known genes related to the development and progression of cancer, and marker genes for chemosensitivity [56]. We were able to identify clusters of genes that differentiated stage grouping (Fig. 3). These lines of evidence indicate that microarray analysis is useful to search for novel prognostic factors, and it also has great potential for identifying the characteristics of individual cancers from the viewpoint of gene expression profiles.

Array-based technology can be applicable to the study of chromosomal aberrations related to tumor progression and prognosis. Microarray comparative genomic hybridization demonstrates the genomic profiling of gastric cancer, and chromosomal copy number changes predict metastatic status and survival [67]. Gain of 1q32.3 is significantly correlated with lymph-node status, while tumors with loss of 18q22.1, as well as tumors with amplifications, are associated with poor prognosis of the patients.

Application of novel prognostic markers in molecular diagnosis

Figure 4 illustrates a strategy for the molecular diagnosis of gastric cancer in pathology samples [56]. The expression of novel prognostic factors can be examined

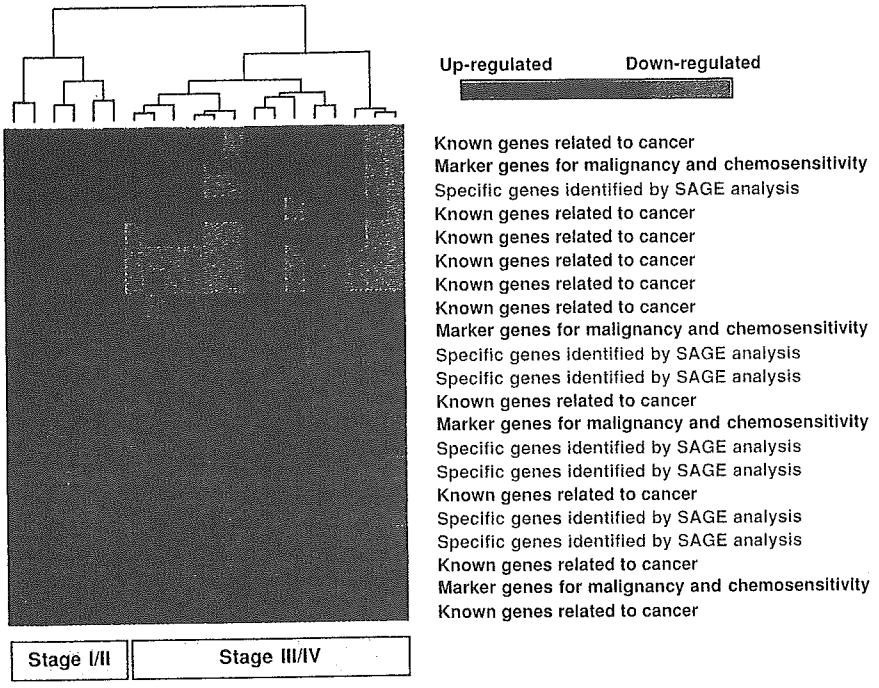

Fig. 3. Hierarchical clustering analysis of gene expression profiles by tumor stage. Gene expression profiles were examined in 20 surgically resected gastric cancer tissues after T7-based RNA amplification, using a mixture of normal gastric mucosal tissues as a reference. Twenty-one genes showing significant correlation with stage grouping were selected, using one-way analysis of variance (ANOVA; $P < 0.05$) by GeneSpring (Silicon Genetics, Redwood, CA, USA), and hierarchical clustering was performed. *SAGE*, Serial analysis of gene expression

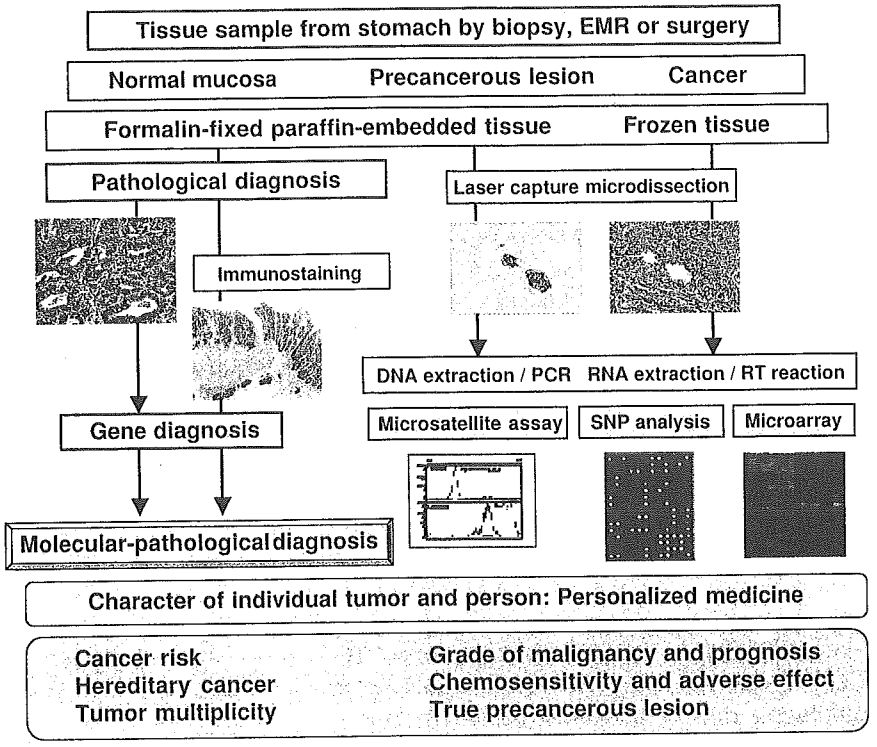

Fig. 4. Strategy for the molecular-pathological diagnosis of gastric cancer using tissue samples. *EMR*, endoscopic mucosal resection; *SNP*, single-nucleotide polymorphism

immunohistochemically if antibodies are available. A system for detection in blood samples can be established using the antibodies. From tissues samples, either freshly frozen or fixed with formalin and embedded in paraffin, RNA or DNA is extracted. The gene expression profile, obtained with a custom-made microarray, gives information on the grade of malignancy/prognosis and chemosensitivity/adverse effects. Microsatellite analysis predicts tumor multiplicity. Analysis of genetic polymorphisms will give information on cancer risk and

sensitivity to chemotherapy, and predict the biological behavior of the cancer. A combination of these examinations can not only foretell the patient's prognosis but can also clarify the characteristics of the individual tumor and person, which are directly connected with genomic medicine; namely, personalized medicine and cancer prevention.

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A Single Nucleotide Polymorphism in the 5′ Untranslated Region of the *EGF* Gene Is Associated with Occurrence and Malignant Progression of Gastric Cancer

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Key Words
Case-control study · *EGF* · Gastric cancer · Single nucleotide polymorphism · 5′-Untranslated region

Abstract
Objective: Epidermal growth factor (EGF) has many biological functions and plays an important role in the progression of various tumors including gastric cancer. An A-G single nucleotide polymorphism (SNP) at position 61 in the 5′-untranslated region (UTR) of the *EGF* gene has recently been reported to be associated with different levels of EGF production. We examined whether this polymorphism is correlated with the development and malignant phenotypes of gastric cancer. ***Methods:*** The study population included 200 gastric cancer patients and 230 healthy control subjects. The SNP in the 5′-UTR of the *EGF* gene was analyzed by polymerase chain reaction-restriction fragment length polymorphism. ***Results:*** The A allele was significantly less frequent in patients than in controls (p = 0.01). Individuals with the A/A or A/G genotype showed a significantly lower risk of gastric cancer than those with the G/G genotype [adjusted odds ratio (OR) = 0.56], whereas the same genotypes were associated with malignant progression of this cancer, e.g. deeper tumor invasion, increased lymph node metastasis and advanced clinical stage, and histological classification in gastric cancer patients (adjusted OR = 1.80, 1.98, 2.26 and 1.89, respectively). ***Conclusions:*** Our findings suggest that the A-G polymorphism of *EGF* is involved not only in the occurrence but also in the malignant progression of gastric cancer.

Introduction

In many countries, the incidence of gastric cancer has declined, probably as a result of changes in environmental factors, especially the diet. Nevertheless, this cancer is still the second leading cause of cancer mortality worldwide [1–3] due to its generally poor prognosis. Gastric carcinogenesis is a multistep process in which genetic and environmental factors interact with each other [4–8]. Environmental factors such as dietary habits, smoking, and *Helicobacter pylori* infection are associated with the risk

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of gastric cancer [2, 8, 9]. Alterations in various genes, including oncogenes, tumor-suppressor genes, DNA repair genes, cell-cycle-related genes and cell-adhesion-related genes, have been implicated in the course of gastric carcinogenesis [10–12].

Epidermal growth factor (EGF) activates multiple signaling pathways by binding with its receptor (EGFR) [13, 14], resulting in proliferation, differentiation and tumorigenesis of epithelial tissues [15, 16]. EGF is also associated with growth and invasion of various malignant tumors by autocrine and paracrine pathways [17–19]. In the gastric mucosa, EGF is involved in pathogenic mechanisms of gastric mucosal hyperproliferation and possibly carcinogenesis in cooperation with *H. pylori* [20]. We have previously reported that EGF works as a potent growth factor for gastric cancer cells in cooperation with interleukin (IL)-1 and IL-6 [21, 22], that expression of EGF and EGFR is upregulated in advanced gastric cancers [23–25] and that patients with synchronous expression of EGF and EGFR have a poor prognosis [23, 26]. Thus EGF is thought to play a pivotal role in the occurrence and malignant progression of gastric cancer.

A recent study of northern Europeans revealed that an A-G single nucleotide polymorphism (SNP) is present at position 61 in the 5′-untranslated region (UTR) of the *EGF* gene and that peripheral blood mononuclear cells from individuals with the A/A genotype produced significantly lower levels of EGF than cells from individuals with the A/G or G/G genotype [27]. Furthermore, it has been reported that the G/G genotype is closely associated with the occurrence of malignant melanoma and its invasive phenotypes [27, 28]; however, another study did not support a significant association between melanoma and the G allele or G/G genotype [29]. In glioblastomas, the A/G and G/G genotypes were associated with more aggressive disease compared to the A/A genotype [30].

We hypothesized that this functionally defined *EGF* polymorphism may be associated with genetic predisposition to the development and malignant progression of gastric cancer. In the present study, we tested this hypothesis in a case-control study and clinicopathological analysis of patients.

Patients and Methods

Study Subjects

This study included a total of 200 patients with gastric cancer who underwent surgery or endoscopic mucosal resection at the Hiroshima University Hospital during the period 1990–2001, at the Hiroshima Memorial Hospital during the period 1998–2000, or at the Hofu Institute of Gastroenterology during the period 2000–2001. We confirmed microscopically that all study patients had gastric adenocarcinoma. The gastric cancers were characterized clinicopathologically according to the TNM classification system [31], and the cancers were classified pathologically as intestinal or diffuse type, as defined by Lauren [32]. We randomly selected 230 healthy control subjects from individuals who visited the three hospitals for regular checkups or because of symptoms such as appetite loss or epigastralgia. Control subjects were confirmed to be free of malignancy by examination with a gastric endoscope and by biopsy. *H. pylori* infection in 94 patients and 125 controls was examined either by histologic examination of endoscopic biopsy samples or by enzyme immunoassay (high titer of anti-*H. pylori* IgG). The characteristics of the 200 gastric cancer patients and 230 controls are summarized in table 1. Written informed consent was obtained from all patients and control subjects prior to enrollment into the study. Moreover, for strict privacy protection, investigators were not able to connect the subjects' identity to the anonymously coded samples. The study was approved by the Human Genome Research Ethics Screening Committee of Hiroshima University School of Medicine.

Table 1. Characteristics of the study subjects

	Controls (n = 230)	Patients (n = 200)
Sex		
Male	108 (47.0%)	142 (71.0%)
Female	122 (53.0%)	58 (29.0%)
Age, years (mean ± SD)	43.9 ± 20.1	65.0 ± 11.6
H. pylori infection		
Positive	81 (64.8%)	61 (64.9%)
Negative	44 (35.2%)	33 (35.1%)
Total	125	94

DNA Extraction

DNA was extracted from peripheral blood samples of 113 gastric cancer patients and 230 control subjects with the QIAamp® 96 DNA Blood Kit (QIAGEN, Valencia, Calif., USA). DNA was extracted from freshly frozen non-neoplastic gastric mucosa of the remaining 87 gastric cancer patients with a genomic DNA purification kit (Promega, Madison, Wisc., USA). We confirmed microscopically that the non-neoplastic mucosa from patients did not show tumor cell invasion or significant inflammatory involvement.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

Genotyping of *EGF* was done by PCR-RFLP as described previously [27]. The target sequence was amplified by PCR from 10–20 ng of genomic DNA in 25 μl of reaction volume containing 200 μ*M* of each deoxynucleotide triphosphate, 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 2 m*M* $MgCl_2$, 0.3 μ*M* each primer, and 0.75 units Ampli*Taq* Gold (Perkin-Elmer, Norwalk, Conn., USA). Amplification conditions were a single cycle of 10 min at 94°C followed by 35 cycles of 30 s at 94°C 30 s at 51°C and 1 min at 72°C, and a final cycle extension of 10 min at 72°C. The PCR primers used were 5′-TGTCACTAAAGGAAAGGAGGT-3′ (EGF/U) and 5′-TTCA-

CAGAGTTTAACAGCCC-3′ (EGF/L) [27]. The 242-bp PCR product, which contained position 61 in the 5′-UTR of *EGF*, was digested with *Alu* I (TAKARA Bio, Shiga, Japan) overnight at 37°C, followed by 8% polyacrylamide gel electrophoresis. *Alu* I digestion yielded 15-, 34-, 91- and 102-bp fragments for the *A* alleles and 15-, 34- and 193-bp for the G allele. Heterozygotes showed a combination of these binding patterns.

Statistical Analysis

Statistical analysis was performed with the χ^2 test. $p < 0.05$ was considered statistically significant. Odds ratios (ORs) and 95% confidence intervals (CIs) were used to estimate the risk of association with genotypes. ORs for the genotypes were calculated by the logistic regression model, with adjustments for age and gender; logistic regression analysis was performed for the association between genotypes and clinicopathological factors (SPSS 11.0, SPSS, Chicago, Ill., USA).

Results

Risk of Gastric Cancer in Relation to the EGF *Genotype*

We first compared the *EGF* genotype and allele frequencies between gastric cancer patients and control subjects. Representative PCR-RFLP patterns of the *EGF* genotypes are shown in figure 1. G/G, G/A and A/A genotypes were observed in 119 (59.5%), 66 (33.0%) and 15 (7.5%) of 200 gastric cancer patients, respectively, and in 108 (47.0%), 97 (42.1%) and 25 (10.9%) of 230 control subjects, respectively (table 2). The genotype distribution among controls was in good agreement with the Hardy-Weinberg equilibrium, although this distribution was different from those of previous reports, possibly because of ethnic differences [27–30]. *A* allele was detected less frequently in gastric cancer patients than in control subjects ($p = 0.01$, table 2). The A/A and A/G genotypes were associated with a lower risk of gastric cancer with ORs of 0.55 (95% CI 0.27–1.09) and 0.62 (95% CI 0.41–0.93), respectively. This lower risk associated with the A/A and A/G genotypes did not change even after adjustment for sex and age. The adjusted OR for A/A was 0.52 with a 95% CI of 0.23–1.21 for A/A, and the adjusted OR for A/G = 0.56 with a 95% CI of 0.35–0.92. Combined genotyping of A/A and A/G revealed a significantly decreased risk of gastric cancer (adjusted OR = 0.56; 95% CI, 0.35–0.89). In addition, the reduced risk was observed in both men and women.

EGF *Genotyping and Clinicopathological Characteristics*

We next analyzed the association between *EGF* genotype and clinicopathological characteristics of gastric cancer. A significant association was observed between the combined A/A and A/G genotypes and aggressive phenotypes for gastric cancer (table 3). The combined genotypes A/A and A/G were found more frequently in T3 and T4 tumors than in T1 and T2 tumors (adjusted OR = 1.80; 95% CI, 0.98–3.30), in N2 and N3 tumors than in N0 and N1 tumors (adjusted OR = 1.98; 95% CI, 1.01–3.89) and in stage III and IV tumors than in stage I and II tumors (adjusted OR = 2.26; 95% CI, 1.21–4.22). The A/A or A/G genotype was found more frequently in the diffuse type tumors than in intestinal type tumors (adjusted OR = 1.89; 95% CI, 1.04–3.45). No statistically significant difference was detected in genotype distribution with respect to the *H. pylori* status in gastric cancer patients.

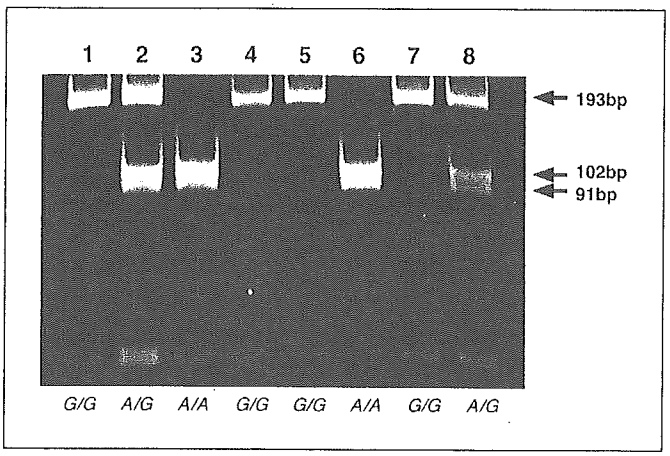

Fig. 1. Representative PCR-RFLP patterns for the A-G SNP in the *EGF* gene. Lanes are individual study subjects; genotypes are indicated below the panel. Digestion with *Alu* I generated fragments of 15, 34, 91 and 102 bp for A/A, fragments of 15, 34 and 193 bp for G/G and fragments of 15, 34, 91, 102 and 193 bp for A/G. Fragments longer than 91 bp were shown.

Discussion

We used a case-control study to examine the association between the A-G polymorphism in the *EGF* gene and the occurrence of gastric cancer. We found that individuals with the A/A or A/G genotype had a significantly lower risk of gastric cancer in comparison to those with the G/G genotype. In a subsequent analysis of the association between the polymorphism and tumor features, patients with the A/A or A/G genotype showed more malignant phenotypes, e.g. deeper tumor invasion, increased lymph node metastasis and advanced clinical stage, being more

Table 2. *EGF* allele frequency and genotype distribution in the study subjects

	Controls (%) (n = 230)	Patients (%) (n = 200)	OR (95% CI) crude	OR (95% CI) adjusted[1]
Allele frequency				
G	313 (69.6)	304 (76.0)		
A	147 (30.4)	96 (24.0)*		
Genotype distribution				
G/G	108 (47.0)	119 (59.5)	1.0	1.0
A/G	97 (42.1)	66 (33.0)	0.62 (0.41–0.93)	0.56 (0.35–0.92)
A/A	25 (10.9)	15 (7.5)	0.55 (0.27–1.09)	0.52 (0.23–1.21)
A/G and A/A	122 (53.0)	81 (40.5)	0.60 (0.41–0.88)	0.56 (0.35–0.89)
Males				
G/G	50 (46.3)	84 (59.2)	1.0	1.0
A/G and A/A	58 (53.7)	58 (40.8)	0.60 (0.36–0.99)	0.72 (0.39–1.31)
Females				
G/G	58 (47.5)	35 (60.3)	1.0	1.0
A/G and A/A	64 (52.5)	23 (39.7)	0.60 (0.32–1.12)	0.40 (0.19–0.86)

The genotype distribution observed in controls was in agreement with the Hardy-Weinberg equilibrium. * p = 0.01 vs. controls (χ^2 test).

[1] ORs were adjusted for sex and age by the logistic regression model.

Table 3. Clinicopathological characteristics according to the *EGF* genotype

Clinical parameters	Category		OR[1] (95% CI) crude	OR[1] (95% CI) adjusted[2]
T grade[3]	T1, T2	T3, T4		
G/G (n = 119)	87 (73.1)	32 (26.9)	1.0	1.0
A/A and A/G (n = 81)	49 (60.5)	32 (39.5)	1.78 (0.97–3.24)	1.80 (0.98–3.30)
N grade[3]	N0, N1	N2, N3		
G/G	98 (82.4)	21 (17.6)	1.0	1.0
A/A and A/G	57 (70.4)	24 (29.6)	1.97 (1.01–3.84)	1.98 (1.01–3.89)
Stage[3]	I, II	III, IV		
G/G	92 (77.3)	27 (22.7)	1.0	1.0
A/A and A/G	49 (60.5)	32 (39.5)	2.23 (1.20–4.13)	2.26 (1.21–4.22)
Histological classification[4]	intestinal	diffuse		
G/G	74 (62.2)	45 (37.8)	1.0	1.0
A/A and A/G	39 (48.1)	42 (51.9)	1.77 (1.00–3.14)	1.89 (1.04–3.45)
H. pylori infection	negative	positive		
G/G	22 (36.1)	39 (63.9)	1.0	1.0
A/A and A/G	11 (33.3)	22 (66.7)	1.13 (0.46–2.78)	1.06 (0.42–2.64)

[1] ORs and 95% CIs for clinicopathological features with reference to the 5′-UTR of the *EGF* gene (A/A+A/G to G/G genotypes).

[2] Adjusted for age and gender, using a logistic regression model.

[3] TNM grades were according to the criteria of the TNM classification [31].

[4] Gastric cancer classified histologically according to the criteria of Lauren [32].

frequent in the diffuse type. This is the first report of an association between the A-G polymorphism of the *EGF* gene and gastric cancer.

It is difficult to give a satisfactory interpretation of our findings at this stage, although the functional significance of this polymorphism has been reported previously: lower levels of EGF are produced by cultured peripheral blood mononuclear cells from individuals with A/A than from individuals with A/G or G/G [27]. It is not clear whether this polymorphism is functional or whether it is closely linked to a different functional polymorphism; however, the polymorphic site does not correspond to any known transcription factor binding site [27]. In the latter, the linkage disequilibrium between this polymorphism and the functional polymorphism may be altered in different ethnic groups. In fact, the frequency of the G allele in Japanese controls was 70% in the present study, which is very different from the 44% in European controls [27] and the 40% in Caucasians [28]. It is also possible that transcriptional regulation specific to the gastric mucosal epithelium or gastric cancer may modulate the association between the A-G polymorphism and EGF production. Nevertheless, it is unlikely that the G/G genotype is associated with a lower production of EGF, because many studies, including ours, have indicated that increased expression of EGF or EGFR is closely associated with more malignant phenotypes [23–25]. Further investigation of the functional significance of this polymorphism in the Japanese population is needed.

Furthermore, the complex roles of EGF in normal gastric mucosa (i.e. cell proliferation, cell differentiation and mucosal protection from injury) make it difficult to give a plausible and consistent interpretation of our finding. The A/A and A/G genotypes showed a decreased risk of gastric cancer, whereas the same genotypes were associated with malignant progression of this cancer. In addition, the EGF ligand/receptor system may require the information of EGFR activation and signaling partners, specifically erbB-2, in gastric cancer among study patients, which we do not have at this moment and will be a future subject of study. Recently, it has been reported that a polymorphic CA repeat is present in intron 1 of the *EGFR* gene, which determines the basal transcription activity of the *EGFR* gene [33–35]. Consequently, malignant behavior may not only be associated with the A-G polymorphism in the *EGF* gene but also with the CA repeat polymorphism in the *EGFR* gene.

Given the potential implications of our findings, further study is warranted to assess the functional significance of this polymorphism in gastric mucosa and cancer. The EGFR status in gastric cancer should be analyzed in combination with this polymorphism with respect to malignant phenotypes.

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Solid Cancer Incidence among Atomic Bomb Survivors: Preliminary Data from a Second Follow-Up

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More than half a century after the atomic bombings in Hiroshima and Nagasaki, an increased risk of cancer incidence is still apparent among the Life Span Study (LSS) cohort of survivors. Although a great deal has been learned from the long follow-up of the LSS cohort, questions regarding radiation-related cancer risks still remain. We are conducting a second comprehensive cancer incidence follow-up to help answer some of these questions. Since the 1987 follow-up, there was a 24% increase in person-years and 56% increase in cancer cases. With the additional 11 years of follow-up, i.e. now including the years from 1958 to 1998, almost 17,500 first primary solid cancers were identified among over 105,000 LSS members with estimated DS02 organ doses.

The LSS cohort includes 120,321 people including about 50,000 survivors who were within 2.5 km of the bombings, about 45,000 who were within 2.5-10 km, and also about 25,000 who were not in either Hiroshima or Nagasaki at the time of the bombings, the so-called Not-In-City (NIC) group. In the past, the NIC group was not included in most of the overall comprehensive studies, but they are included in the second follow-up because they can improve inference about baseline risk patterns.

There are several important strengths of the LSS cohort. It is a large, healthy non-selected population that includes all ages and both sexes (though there are more females due to the fact that many male soldiers were not in the cities of Hiroshima and Nagasaki); members were exposed to a wide range of doses and they have well characterized dose estimates; mortality follow-up is virtually complete since 1950; cancer incidence ascertainment is complete in Hiroshima and Nagasaki tumor registry catchment areas since the establishment of the registries in 1958, and there is more than 50 years of follow-up.

When studying cancer incidence or mortality, certain differences in methods should be noted. For evaluating cancer incidence, we must exclude people who either died or had cancer diagnosed before the cancer registries were established in 1958. Therefore, there are about 8,000 fewer people in incidence analyses than in mortality analyses. Also, the mean age at the time of the bombing is a little younger in the survivors included in the incidence (26.8 years) compared with mortality (29.0 years) analyses because people who developed cancer before 1958 tended to be old and, as already mentioned, they are excluded from the incidence analyses.

Cancer incidence ascertainment is based on the LSS Tumor Registry. This registry includes all cancer cases diagnosed among LSS members registered in either the Hiroshima or Nagasaki Tumor Registries. The Hiroshima and Nagasaki Tumor Registries are of high quality because they employ active case identification in all large hospitals in their catchment areas. Data from tissue registries, death certificates, and medical associations (for the small hospitals) are also collected. Earlier analyses demonstrated that there is no dose bias in case ascertainment. Mortality data are obtained from the family registry (called Koseki) and they are nationwide.

The LSS cancer incidence studies add a valuable component to radiation risk assessment of the atomic bomb survivors because they include data on non-fatal cancers, some of which are quite radiation sensitive. Cancers of the breast, thyroid and skin, for example, are radiation sensitive but since they have very good survival a large number of them would be missed if only mortality data were evaluated. The incidence data are characterized by a high level cancer ascertainment, accurate diagnoses, information on histology, and long follow-up. For some organs, information on benign tumors also is collected.

The LSS cancer incidence studies do have some limitations. In particular, solid cancer data from 1945 to 1958 and leukemia data from 1945 to 1950 are incomplete, cancer ascertainment is limited to Hiroshima and Nagasaki area residents, and treatment data are limited. This means that some early cancer cases have been missed, especially leukemia and thyroid cancers which have a short latency period.

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The second comprehensive cancer incidence report includes follow-up from 1958 to 1998, with data on 105,427 people; 50% of whom were still alive in 1998 (currently about 45% are alive). Of note is that about 85% of individuals less than 20 years of age at the time of bombing were still alive in 1998 (about 80% today). In this report, we are studying only first primary tumors to prevent confounding from radiation treatment for the first cancer and possible detection bias in persons who already have cancers. All analyses in this report are based on the new DS02 dosimetry system which has incorporated several important improvements over DS86. Improvements in DS02 include refinements in the shielding calculations, transport calculations, and source term adjustment. In DS02, gamma doses increased and neutron doses decreased slightly. We used weighted colon dose in Gy to evaluate solid cancer and weighted organ doses for most site-specific analyses.

Table 1 shows the study population by dose categories. Excluding the non-exposed NIC group, 35,545 (slightly over 44% of the 80,180 exposed LSS members) A-bomb survivors were exposed to less than 0.005 Gy and 63,334, or 79% of the exposed cohort, were exposed to less than 0.1 Gy. Thus, the LSS is not such a high dose study as some may think, and it can provide substantial information on low dose radiation.

We used Poisson regression analysis to estimate the excess relative and absolute risks of all solid cancers combined and of individual cancer sites. The excess relative risk (ERR) quantifies the percentage change in risk for a unit of dose, in this case in Gy, i.e. it shows the relative change in cancer rates. The excess absolute rate (EAR) quantifies the absolute change in rates for a unit of dose, i.e. it shows the difference in cancer rates. The ERR and EAR can vary with age at exposure, gender, attained age, and other factors. They are both important and provide complementary information. In the analyses, we adjusted the person years of follow-up for the estimated migration of persons out of the Hiroshima and Nagasaki areas. We used a linear dose-response model as our standard, and considered the modifying effects of gender, attained age, age at exposure, and time since exposure.

In the second follow-up, 17,448 cancers were identified among the LSS cohort members (Table 2). The largest group of tumors (n=10,052) is of the digestive system, and stomach cancer which is a very common cancer in Japan was the most frequent cancer of the digestive tract. There were over 1000 cancer cases of the respiratory system, female genital organs, and breast cancer.

For all solid cancers combined, the dose response was linear and we saw no evidence of non-linearity. A statistically significant dose response trend was seen in the 0 - 0.15 Gy range, and this trend was consistent with that observed for the full dose range. The ERR per weighted colon dose in gray (ERR/Gy) for solid cancer was higher for women than men and decreased with increasing age at exposure and attained age. The EAR per 10,000 person years per weighted colon dose in Gy (EAR/10^4 PY Gy) was also higher among women and decreased with increasing age at exposure, but increased with increasing attained age. When gender-specific cancers were excluded from the analyses, the ERR/Gy remained significantly higher for females than males, but the gender difference disappeared when an absolute risk model was used. Lifetime solid cancer risk estimates appear to be about 20 times higher than those observed for leukemia.

Table 1. Dose distribution in the LSS incidence cohort

Dose (Gy)	Number of Subjects	Percentage (%)
Not in city	25,247	23.9
< 0.005	35,545	33.7
0.005 - 0.1	27,789	26.4
0.1 - 0.2	5,527	5.2
0.2 - 0.5	5,935	5.6
0.5 - 1	3,173	3.0
1 - 2	1,647	1.6
2+	564	0.5
Total	105,427	100

Table 2. Distribution of solid cancers identified among the LSS cohort members during the period of 1958-1998

Site	Number of subjects
Digestive system	10,052
Respiratory system	2,001
Female genital	1,457
Breast	1,082
Urinary system	741
Thyroid	471
Skin	347
Male genital	420
Oral cavity	277
Nervous system	281
Other solid cancers	319
Total	17,448

As a result of the second follow-up, there is now a suggestion of an excess relative risk for endometrial cancer among women exposed before age 20. We also have identified radiation effects for male breast cancer, and found strong evidence that some time patterns differ when using the ERR and the EAR models. Using an EAR model, risk increased with increasing age, whereas the risk decreased with an ERR model.

Patterns of organ (or site) specific risks generally were similar to those seen in the previous follow-up, but the risk patterns have become clearer for some cancers. High ERRs were found for cancers of the bladder, breast and lung, while high EARs were seen for cancers of the stomach, breast, colon and lung. Assessing site-specific cancer risks is important, but because there are considerably fewer cases, it is difficult to identify significant differences in risk estimates or patterns. Biologically it is almost certain that variation in site-

specific risks exists, while current analyses suggest some differences much of the observed variability is consistent with random variation because formal statistical tests generally lack the power to detect real differences.

In summary, the updated solid cancer incidence data indicate that the shape of the dose response is well described by a linear model. Solid cancer excess rates increased throughout life for all ages, while excess relative risks decreased with increasing age. Excess risks for all solid cancers were higher for women than men, and lifetime risk estimates were considerably larger than for leukemia. The relatively small number of cancers for most individual sites made it difficult to identify statistically significant differences in age-time patterns. While overall patterns were similar to those seen in previous analyses, we continue to find new results with each new follow-up.

A large proportion of the radiation-associated excess solid cancers are likely to occur over the next 15 to 20 years. We therefore expect that the accumulating data will continue to offer important new insights into radiation effects on cancer risks. Continued follow-up is necessary to understand risk patterns for persons less than age 20 years at the time of the bombings. Additional site-specific incidence studies incorporating pathological reviews will provide needed information on the radiation-sensitivity of specific histologies. With close collaboration among statisticians, epidemiologists, biologists and pathologists; we should be able to improve our understanding of these data and their implications for radiation protection.