tended to be poor on follow-up observation. About 4% of gastric cancers were judged to have high-frequency microsatellite instability (MSI-H) by microsatellite assay, and half of the patients showing MSI-H were confirmed to have clinically synchronous or asynchronous multiple primary cancers. Detailed methods and results were described elsewhere.[3,9] This system may be applicable to molecular diagnosis in the future if appropriate molecular and genetic markers are selected.

## DNA Methylation–Targeted Molecular Diagnosis

DNA methylation is the most important event among various epigenetic alterations in cancers. To regulate gene expression at the transcriptional level, DNA methylation, histone modification, and chromatin remodeling function as an on–off switch, while transcription factors act as a volume switch.[10] Hypermethylation of CpG islands is associated with gene silencing of many tumor suppressors, including hMLH1, p16, CDH1 (E-cadherin), RAR-β, RUNX3, and $O^6$-methylguanine-DNA methyltransferase (MGMT) in gastric cancer. Aberrant DNA methylation is readily detected in cancer-derived DNA in the serum of patients with gastric cancer. Many reports have indicated that aberrant DNA methylation is a useful diagnostic marker and a prognostic indicator.[11-14] DNA methylation of p16 and of CDH1 was detected in the serum of 20% to 50% of gastric cancer patients, whereas none of the controls without cancer showed aberrant methylation.[11-14] Some of the DNA methylation is associated with tumor stage and prognosis. For instance, gastric cancer patients with CDH1 methylation showed significantly poorer prognosis than those without aberrant methylation[15] RUNX3 methylation was detected in the peripheral circulation of 30% of gastric cancer patients and was concordant with tumor stage and lymphatic and vascular invasion.[16] After surgical removal of gastric cancer, RUNX3 methylation in serum decreases significantly. Detection of aberrant DNA methylation in serum is a useful and effective tool in cancer screening, monitoring, and prognosis.

Infection with HP, a potent gastric carcinogenic factor, has been shown to induce aberrant DNA methylation in gastric mucosa and produce a predisposed field of cancerization.[17] Methylation levels of p16, LOX, FLNc, HRASLS, HAND1, THBD, and p41ARC in gastric mucosa are higher in HP-positive individuals than in HP-negative persons among healthy volunteers.[18] Among HP-negative individuals, methylation levels in non-neoplastic mucosa are higher in gastric cancer cases than in controls.[19,20] Furthermore, significant increasing levels of methylation are present in the following order: healthy volunteers, single gastric cancer cases, and multiple gastric cancer cases. Among HP-positive individuals, methylation levels are highly variable. The evidence indicates that the measurement of methylation levels among individuals without current HP infection is a promising risk marker for gastric cancer and can be used in molecular diagnosis to predict future risk of gastric cancer.

Endoscopy followed by pathologic examination has been proven to be useful for the detection and diagnosis of gastric cancer; however, the diagnostic power depends on the technical skill of the endoscopist, although the sensitivity and specificity are generally high. Detection of molecular markers in stomach juice or gastric washes is a possible non-invasive approach to screen for gastric cancer. There is evidence that methylation analysis of DNA recovered from gastric washes can be used to detect gastric cancer.[21] The methylation status of six genes (*MINT25*, *RORA*, *GDNF*, *ADAM23*, *PRDM5*, and *MLF1*) in gastric washes differs significantly between patients with gastric cancer and those without. *GDNF* and *MINT25* are the most sensitive molecular markers of early cancer, while *PRDM5* and *MLF1* are potential markers of epigenetic field defects. There is a close association between the methylation levels in tissue samples and gastric washes. *MINT25* methylation in gastric washes shows the best sensitivity (90%) and specificity (96%) and may have value as a powerful molecular tool for screening of gastric cancer. Genomic variations of HP can be analyzed in samples recovered from gastric washes.[22] Antibiotic-resistant HP is correlated with 23S rRNA single-nucleotide polymorphisms (SNPs). Gastric wash–based PCR and pyrosequencing are useful for detecting SNPs and diagnosing drug resistance.

## Molecular Diagnosis for Molecular-Targeted Therapy

Molecular-targeted therapy refers to drugs (inhibitor or monoclonal antibody) that selectively inhibit specific molecular pathways that are involved in the development, progression, and metastasis of cancers. A number of biologic agents modulating different signaling pathways are currently in clinical development, such as agents targeting angiogenesis, growth factor receptor, cell cycle regulator, matrix metalloproteinase, and mammalian target of rapamycin (mTOR).[23-25] Several randomized multicenter phase III studies are underway in molecularly unselected patients with gastric cancer, considering cetuximab for EGFR, lapatinib for EGFR/HER2, panitumumab for EGFR, everolimus for mTOR, and ramucirumab for VEGFR-2. Furthermore, a stromal cell–targeted strategy, such as anti-stromal therapy with imatinib for PDGFR, has been advocated.[26]

To date, only trastuzumab, an anti-HER2 monoclonal antibody, has been approved for use in combination with chemotherapy to treat HER2-positive advanced gastric and esophago-gastric junction cancers, on the basis of the results of the ToGA (Trastuzumab for Gastric Cancer) trial.[27] Molecular diagnosis of HER2 status is made by immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) or dual-color silver-enhanced in situ hybridization (DISH). DISH is an alternative to FISH

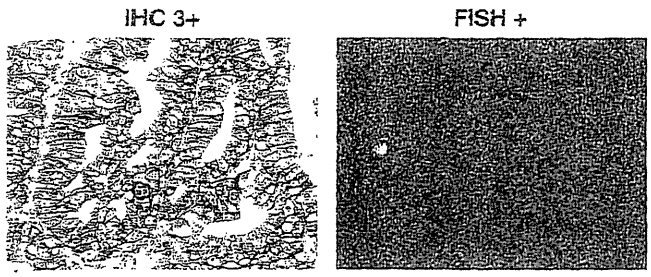


**FIGURE 29–2** Evaluation of HER2 in gastro-esophageal junctional and gastric adenocarcinoma. **A:** Immunohistochemistry reveals diffuse and strong membranous expression of HER2 protein in a gastric cancer. **B:** Fluorescence in situ hybridization confirms amplification of the *HER2* gene (*red signal*), indicating this patient may benefit from targeted therapy of Herceptin.

with the same accuracy (concordance rate is 97%), and signals can be observed under conventional light microscopy.[28] Tumors showing as IHC 3+ (strong membrane staining in over 10% of cancer cells) and FISH/DISH positive (HER2:CEP17 ratio>2) are regarded as HER2 positive (Fig. 29.2) and subjected to trastuzumab therapy. Because HER2 status in gastric cancer shows marked heterogeneity, it is recommended that the order of testing for HER2 is IHC followed by FISH/DISH. IHC 2+ (moderate membrane staining in >10% of cancer cells) is judged as borderline or equivocal, and subsequent HER2 amplification should be confirmed by FISH/DISH. Total HER2-positive rate is 12% to 18%, with 20% to 30% as the differentiated type and 3% to 6% as the undifferentiated type. However, evaluation of HER2 status by this method may not be definitive for patient selection, because not all HER2-positive patients respond to trastuzumab treatment.

## Molecular Diagnosis of Micrometastasis and Circulating Tumor Cells

Lymph node metastasis is an important determinant of patient outcome. Routine pathologic examination of representative sections of the cut surface may overlook micrometastasis. Molecular detection of mRNAs for cytokeratin (CK) 19 and CEA by RT-PCR is useful for detecting micrometastasis. In recent years, the sentinel lymph node concept has been validated for gastrointestinal cancer in addition to breast cancer.[29] The sentinel node is defined as the first node to receive lymphatic drainage from the primary tumor. According to this concept, lymph node dissection can be avoided if no metastases are detected in the sentinel node. Therefore, examination for micrometastasis in the sentinel node must be made intraoperatively during sentinel node navigation surgery. The real-time multiplex RT-PCR assay for the expression of CK19, CK20, and CEA is more sensitive and accurate than histopathologic diagnosis and generates results within 80 minutes intraoperatively.[30] A more rapid molecular diagnosis system has been developed using one-step nucleic acid amplification (OSNA).[31,32] The OSNA system consists of homogenization of lymph node tissue followed by a reverse transcription loop–mediated isothermal amplification (RT-LAMP) and the quantification of a target mRNA, CK19, directly from the lysate. RT-LAMP measurement of CK19 mRNA is performed using an RD-100i gene amplification detector (Sysmex, Kobe, Japan). The whole procedure takes approximately 30 minutes to obtain a final result. The OSNA system is effective and efficient for intraoperative molecular diagnosis during sentinel node navigation surgery in gastric cancer.

Circulating tumor cells (CTCs) are considered a reflection of tumor aggressiveness because hematogenous spreading of CTCs from a primary tumor is a crucial step in the metastatic cascade leading to the formation of metastatic tumor.[33] Molecular methods can detect CTCs in blood with high sensitivity and specificity and can be a useful tool for judging tumor stage, predicting distant metastasis and patient survival, and monitoring the response to cancer therapy.[34] Although clinical relevance remains to be verified in large-scale clinical trials, many detection methods have been established including the above-mentioned RT-PCR–based method.[33] The detection of free cancer cells in the peritoneal cavity has important therapeutic and prognostic implications. The RT-PCR–based technique with the same markers as CTCs, such as CK19 and CEA, is useful for detecting free cancer cells in peritoneal lavage fluid.[35] The clinical significance of establishing the presence of peritoneal metastasis has been assessed by several studies and most have confirmed the predictive value of molecular detection of peritoneal metastasis and recurrence using peritoneal washes.[35]

## miRNA-based Molecular Diagnosis

The role of miRNA in cancer development and progression through epigenetic gene regulation has become a recent focus in cancer research.[6,7] Mature RNAs are composed of 19–25 nucleotides that are cleaved from 60- to 110-nucleotide pre-miRNA precursors by RNase III Dicer.[36] Single-stranded miRNAs bind through partial sequence homology to the 3′-untranslated region of potentially hundreds of target genes and cause degradation of mRNAs and inhibition of translation. miRNAs possess either anti-tumorigenic or oncogenic properties depending on the target genes.[37] In gastric cancer, many miRNAs are expressed differentially, and unique miRNAs are associated with the development, progression, and prognosis of gastric cancer by modulating several biologic pathways.[38] By miRNA microarray analysis, 22 miRNAs were upregulated and 13 were downregulated in gastric cancer in comparison with corresponding non-neoplastic gastric tissue.[39] miR-125b, miR-199a, and miR-433 are important miRNAs involved in cancer progression, while low expressions of let-7g and miR-433 and high expression of miR-214 are independent

unfavorable prognostic markers. miR-146a targeting EGFR and interleukin-1 receptor–associated kinase (IRAK1) is an independent prognostic factor in gastric cancer.[40] miR-148a functions as a tumor metastasis suppressor in gastric cancer, and downregulation of miR-148a contributes to lymph node metastasis and progression.[41] Furthermore, miR-335 also acts as a metastasis suppressor in gastric cancer by targeting Bcl-w and specificity protein 1 (SP-1).[42] The metastasis-associated miR-516a-3p appears to be a potential therapeutic target for inhibiting peritoneal dissemination of scirrhous-type gastric cancer.[43] miR-486 targets olfactomedin 4 (OLFM4), and genomic loss and downregulation of miR-486 are associated with gastric cancer progression.[44] Downregulation of miR-125a-5p targeting HER2 is associated with invasion, metastasis, and poor prognosis of gastric cancer, and its growth inhibitory effect is enhanced in combination with trastuzumab.[45] Therefore, these miRNAs as tumor biomarkers are potential targets for molecular diagnosis in gastric cancer.

As in protein-coding genes, miRNA genes are also transcriptionally regulated by DNA methylation and chromatin remodeling. DNA methylation of *miR-34b* and *miR-129* genes causes downregulation in gastric cancer and is associated with poor clinicopathologic features.[46] In stomach carcinogenesis, a mucosal field with HP infection is a condition predisposing to cancer development. Methylation levels of three miRNA genes (*miR-124a-1*, *miR-124a-2*, and *miR-124a-3*) are higher in gastric mucosa with HP infection than in gastric mucosa without HP infection, and the methylation levels are higher in non-cancerous gastric mucosa taken from gastric cancer patients than in those from healthy individuals.[47] Methylation-associated silencing of miR-34b and miR-34c is detected in a majority of gastric cancers, and the methylation levels are higher in gastric mucosa from patients with multiple gastric cancers than in mucosa from patients with single gastric cancer or mucosa from HP-positive healthy individuals.[48] Thus, methylation-associated silencing of miRNAs contributes to the formation of field defects and may serve as a predictive marker of gastric cancer risk.

miRNAs are stable in human circulation in a cell-free form and may be a powerful new class of blood-based biomarkers for gastric cancer.[49] Stability of extracellular miRNAs is believed to be due to vesicular encapsulation in exosomes and/or binding with argonaute 2 (Ago2) to make Ago2–miRNA complexes.[49] By genome-wide serum miRNA expression profiling, five miRNA signatures (miR-1, miR-20a, miR-27a, miR-34a, and miR-423-5p) for gastric cancer diagnosis have been identified.[50] The levels of the five miRNAs in serum assessed by quantitative RT-PCR are correlated with tumor stage, and the sensitivity and specificity of gastric cancer detection by the five miRNAs as biomarkers are 80% and 81%, respectively. The plasma concentrations of miRNAs (miR-17-5p, miR-21, miR-106a, and miR-106b) are significantly higher in gastric cancer patients than in non-cancerous controls, and the plasma concentrations of these miRNAs are reduced after surgery.[51]

## Diagnosis of Gastric Cancer Risk and Chemotherapeutic Efficacy

Stomach carcinogenesis is modulated by such genetic polymorphisms as mucosal protection by HP infection inflammatory response, carcinogen detoxification and antioxidant protection, DNA damage repair, and cell proliferation. Therefore, genetic polymorphisms are increasingly used for molecular assessment of gastric cancer risk.[6,52,53] Variations of IL-1β (*IL1B*) and IL-1 receptor antagonist (*IL1RN*) genes affect IL-1β production and gastric acid secretion, causing an increased risk of chronic hypochlorhydric response to HP infection and gastric cancer risk.[54] Upon HP infection, CagA in gastric epithelial cells interacts with src homology 2 domain–containing protein tyrosine phosphatase (SHP-2) and transduces signal to downstream molecules participating in atrophic gastritis and stomach carcinogenesis.[55] Frequent G/A SNP in the intron 3 of the *PTPN11* gene encoding SHP-2 is associated with gastric atrophy in the Asian population.[53] Genetic polymorphisms significantly associated with gastric cancer risk include cyclin D1, CDH1, EGFR, $p16^{INK4A}$, $p21^{WAF1/CIP1}$, and HER2.[53] A genome-wide study using Japanese and Korean cohorts found that genetic variation in prostate stem cell antigen (PSCA) is associated with susceptibility to diffuse-type gastric cancer.[56] Furthermore, the same group recently found that *MUC1* is the second major susceptibility gene for diffuse-type gastric cancer, and the SNPs (rs2070803 and rs4072037) in the *MUC1* gene might be used to identify individuals at risk for this type of gastric cancer.[57]

Genetic polymorphisms are also associated with therapeutic efficacy and toxicity of anti-cancer drugs. Pharmacogenomics in gastric cancer has provided a number of putative biomarkers and genetic polymorphisms for the prediction of tumor response to chemotherapies and for prediction of toxicity, and these are summarized in the review by Nishiyama and Eguchi.[58] Polymorphisms of thymidylate synthetase (TYMS) and variation of glutathione-*S*-transferase pi 1 (GSTP1) are associated with responses to the 5-FU–based regimen and platinum-containing therapy, respectively, in gastric cancer. Nucleotide excision repair modulates platinum-based chemotherapeutic efficacy by removing drug-produced DNA damage. Polymorphisms of excision repair cross-complementing 1 (ERCC1) (rs11615C > T) and ERCC2 (rs13181T > G) predict clinical outcomes of oxaliplatin-based chemotherapy in gastric cancer and are useful prognostic factors.[59] In regard to chemotherapeutic toxicity, among the various polymorphisms in the dihydropyrimidine dehydrogenase (*DPYD*) gene, exon 14-skipping mutation (DPYD*2A) is a prominent genotype marker

related to deficiency of enzyme activity resulting in severe toxicity caused by 5-FU–based therapy. Another important consideration regarding drug toxicity is the polymorphism of UDP-glucuronosyltransferase 1A1 (*UGT1A1*) gene (UGT1A1*28), which reduces enzyme activity and results in irinotecan toxicity, especially neutropenia. Molecular examination of such genetic polymorphisms is directly connected to personalized cancer prevention and treatment.

## Novel Molecular Markers Identified through Transcriptome Dissection

Many molecules and genes have been identified as novel diagnostic and therapeutic targets in patients with gastric cancer through transcriptome dissection by using microarray and other techniques. Serial analysis of gene expression (SAGE) is a powerful tool for the global analysis of gene expression in a quantitative manner.[60,61] A comparison of SAGE data between gastric cancers and systemic normal tissues in combination with quantitative RT-PCR, IHC, and biologic studies has identified many genes, including regenerating islet–derived family, member 4 (*Reg IV*) and *OLFM4*, as candidate diagnostic markers and therapeutic targets.[62,63] *Reg IV* is expressed in about 30% of gastric cancers, and Reg IV protein is detectable in sera of about 30% of gastric cancer patients.[64] Reg IV, induced by CDX2, participates in 5-FU resistance in gastric cancer. CDX2 also induces multidrug resistance 1 (*MDR1*) gene, resulting in resistance to chemotherapy.[65] There is an intestinal phenotype of gastric cancer defined by the expression of MUC2, CDX2, and/or CD10. Molecular detection of intestinal phenotype predicts chemoresistance in gastric cancer. OLFM4 is detected in 60% of gastric cancers, with a significant association with the gastric phenotype defined by the expression of gastric-type mucins such as MUC5AC and MUC6.[66] Combined measurement of Reg IV and OLFM4 protein levels in sera shows a sensitivity of 57% and specificity of 95% for detecting gastric cancer. *MMP-10* is one of the cancer-specific genes identified by SAGE data analysis, with serum *MMP-10* diagnostic sensitivity and specificity of 94% and 85%, respectively, indicating that *MMP-10* is suitable for gastric cancer screening.[67] CLDN18 (encoding claudin-18, a major component of tight junction in the stomach) is reduced in HP-positive atrophic gastritis, intestinal metaplasia, gastric adenoma, and the intestinal phenotype of gastric cancer.[68] CLDN18 knockout mice show atrophic gastritis and spasmolytic polypeptide–expressing metaplasia (SPEM) through paracellular $H^+$ leakage, upregulation of proinflammatory cytokines, and loss of parietal cells.[69] Therefore, the detection of loss of CLDN expression is a predictive marker of a precancerous condition. Cell surface and secreted proteins are potential drug targets and tumor markers when they are overexpressed in cancer. The CAST (*Escherichia coli* ampicillin trap) method systemically and efficiently detects gene expression profiles encoding transmembrane and secreted proteins. By this method, several genes that are upregulated in gastric cancer have been identified, including desmocollin 2 (*DSC2*).[70] Because *DSC2* expression is induced by *CDX2* and is significantly associated with the MUC2-positive intestinal phenotype, *DSC2* is also a novel diagnostic marker for chemoresistant gastric cancer of the intestinal phenotype. In short, information obtained from transcriptome dissection greatly contributes to our understanding of the molecular characteristics of gastric cancer and will be connected to new developments in diagnosis and treatment.

## Molecular Testing to Assess Hereditary Gastric Cancer Syndrome

It is estimated that about 10% to 15% of gastric cancers are familial, though the majority of gastric cancers are classified as sporadic. Among the familial gastric malignancies, hereditary diffuse gastric cancer (HDGC) syndrome is the most important condition that leads to familial gastric cancer. Other hereditary cancer syndromes, such as hereditary non-polyposis colorectal cancer, familial adenomatous polyposis, Peutz-Jeghers syndrome, Li-Fraumeni syndrome, and hereditary breast and ovarian cancer, are also associated with a significantly higher risk compared with the general population for developing gastric cancer.[71-73] HDGC patients typically present with diffuse-type signet-ring cell gastric cancer. In addition to a high susceptibility of developing gastric cancer, HDGC patients also have an increased risk of lobular breast carcinoma.

It is now known that germline mutations of *CDH1* gene, encoding E-cadherin, plays an essential role in HDGC.[71-75] Specifically, *CDH1* germline mutations occur in approximately 30% to 40 % of HDGC and missense mutations, such as c.1748T>G(p.Leu583Arg), are frequent. *CDH1* mutation has also been found to have synergistic effect along with other genetic alterations. To examine the synergistic effect of the loss of E-cadherin and p53 on gastric cancer development, a mouse line was established in which E-cadherin and p53 were specifically inactivated in the gastric parietal cell lineage.[71] Mouse diffuse gastric cancer developed at 100% penetrance within a year, frequently associated with lymph node metastasis. Gene expression profiling study of diffuse gastric cancer in DCKO mice resembled those of human HDGC. In addition, the mesenchymal markers and epithelial-mesenchymal transition-related genes were highly expressed in mouse diffuse gastric cancer as in human HDGC. Thus, genetically engineered mouse model of diffuse gastric cancer is very useful for clarifying the mechanism underlying gastric carcinogenesis and provides potentially a novel approach to management of HDGC.

The penetrance of *CDH1* is 70% to 80%, and the average age for the diagnosis of gastric cancer is 37 years.

Currently, there is no consensus regarding who should be tested for *CDH1* mutation.[75] Though age is an important factor, it has been reported that the age at onset and aggressiveness of gastric carcinoma is highly variable, which has to be included in counseling on mutation testing and potentially planning prophylactic gastrectomies.[72] Since early gastric cancer of HDGC is not readily identified by endoscopic examination, prophylactic gastrectomy is the sole preventive treatment for *CDH1* mutation carriers. Because of the occult nature of this special type of specimen, examination of the entire mucosa of prophylactic gastrectomy specimens is essential. Analysis of prophylactic gastric resection specimens has led to the detection of in situ signet-ring cell carcinomas. Usually multiple (20 to >100) foci of in situ signet-ring cell carcinoma (SRCC) and invasion in the superficial lamina propria can be detected by careful examination (Fig. 29.3). Frequently, the foci of carcinoma are very small (Fig. 29.4), and cytokeratin and PAS staining may highlight the signet-ring cells and facilitate the diagnosis.[76]

It is reported that SRCC arises from the upper isthmus of the neck region of the gastric mucosa.[72] In addition, de novo germline *CDH1* mutation is reported in an HDGC kindred presenting with early-onset diffuse gastric cancer.[74] The incident case was a woman with a personal history of Hodgkin lymphoma and diffuse gastric cancer, who was then confirmed to have a germline mutation in *CDH1* (c.1792 C > T (R598X)). The patient's mother had the same *CDH1* germline mutation, while neither maternal grandparent was found to carry this mutation, indicating that the proband's mother's mutation is of de novo origin. This report highlights the importance of recognition of the HDGC syndrome and of testing for *CDH1* germline mutations in young individuals with diffuse gastric cancer without a family history of the disease.

Does *CDH1* represent a target for treatment of HDGC? This is another area with several studies.[71,73] Using cells stably expressing WT E-cadherin and two HDGC-associated missense mutations, a recent study

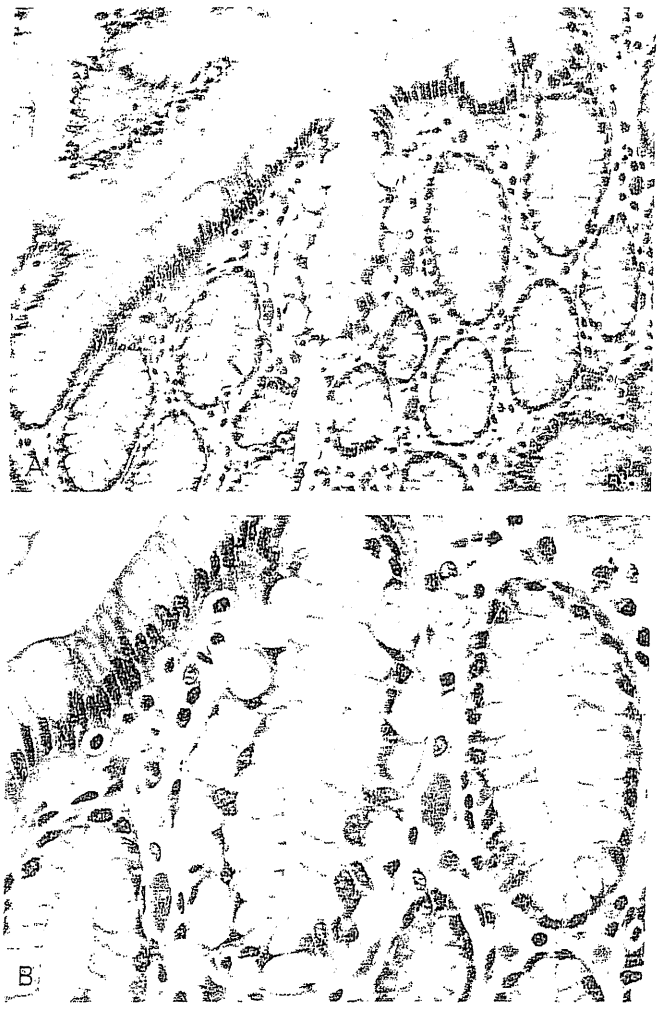


**FIGURE 29–3** In situ signet-ring cell carcinoma in the superficial epithelium of a gastrectomy specimen from a CDH1 mutation carrier. A: Serial sections of a representative SRCC (*arrowed*) at intermediate power magnification (×200). B: Higher magnification of Figure 29.3A (×400).

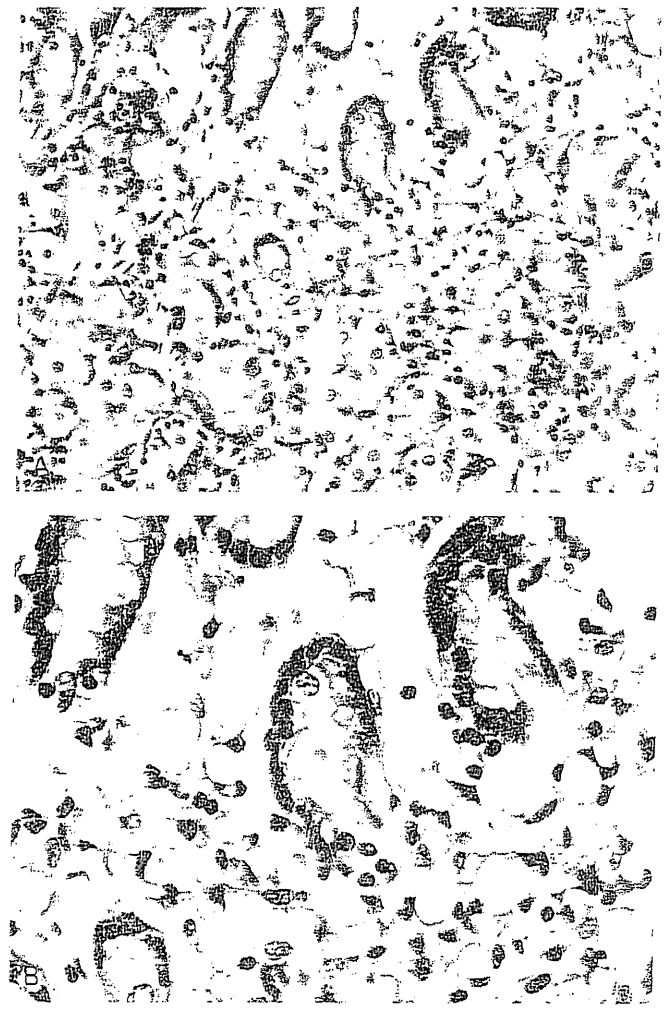


**FIGURE 29–4** Invasive diffuse gastric carcinoma with signet-ring cell morphology from a gastrectomy specimen from a CDH1 mutation carrier. A: Notice that the size of the invasive focus is small (*arrowed*), frequently <0.5 mm in size, and located in the superficial lamina propria, intermediate magnification (×200). B: Higher magnification of Figure 29.4A (×400).

shows that upon DMSO treatment, mutant E-cadherin can be restored and stabilized at the cell membrane, which is associated with altered expression of Arf6 and PIPKIr.[73] Modulation of Arf6 expression partially mimics the effect of chemical chaperones (CCs), indicating that the cellular effects observed upon CCs treatment are mediated by Arf6. The investigators concluded that direct influence of CCs in cellular trafficking machinery and its effects are of crucial importance in the context of juxtamembrane E-cadherin missense mutations associated with HDGC, and they proposed that this influence should be considered when exploring the therapeutic potential of this type of chemicals in genetic diseases associated with protein misfolding.[73]

## MOLECULAR DIAGNOSIS OF ESOPHAGEAL CANCER

### General Concept of the Molecular Basis of Esophageal Cancer

The principle of molecular diagnosis of esophageal cancer is the same as that of gastric cancer. There are differences and similarities in the genes and molecules involved in the development and progression between gastric cancer and esophageal cancer. Furthermore, among esophageal cancers, SCC and adenocarcinomas preceded by Barrett esophagus also show distinct and similar molecular characteristics that are important in molecular diagnosis for the selection of biomarkers suitable for each histologic type of esophageal cancer.[1,77,80] Esophageal SCC develops in sequential steps through normal squamous epithelium, basal cell hyperplasia, dysplasia, and SCC, whereas esophageal adenocarcinoma is believed to develop through the dysplasia–carcinoma sequence in Barrett esophagus, by serial accumulation of genetic and epigenetic alterations.[79]

### Genetic and Epigenetic Alterations in Esophageal Cancer and Diagnostic Implications

In esophageal SCC, mutation of the *p53* gene occurs at an early stage of carcinogenesis and is found in 40% to 60% of SCC and less commonly in non-cancerous mucosa adjacent to the cancer.[77,78] EGFR overexpression, partly due to gene amplification, is correlated with tumor progression, minimal response to chemotherapy, and poor prognosis. Gene amplification and overexpression of cyclin D1 are detected in 25% to 50% of esophageal SCC and cyclin D1 is an independent prognostic marker confirmed by multivariate analysis.[77,78] Homozygous deletion and hypermethylation of the $p16^{INK4a}$ gene are found in 50% to 60% of esophageal SCC and cause dysregulation of the $G_1$/S checkpoint and abnormal proliferation, resulting in metastasis and poor prognosis.[79] Loss of FHIT expression occurs even in normal-appearing squamous epithelium that has been heavily exposed to environmental carcinogens such as tobacco and alcohol.

In esophageal adenocarcinoma, p53 mutation is also an early event in carcinogenesis, as it is detected in Barrett esophagus and dysplasia.[77,80] Alterations in transcription of FHIT and $p16^{INK4a}$ also occur in adenocarcinoma at an early stage. HER2 amplification and overexpression are found in 20% to 30% of esophago-gastric and Barrett esophagus–related adenocarcinomas. In Barrett esophagus and esophageal adenocarcinoma, lack of p27 expression is associated with malignant transformation and poor prognosis.[80]

In regard to epigenetic alterations in esophageal SCC, genes including *APC*, *CDH1*, $p16^{INK4a}$, *RAR*β, and Ras association domain family protein 1 (*RASSF1A*) are highly methylated.[79] CDH1 methylation is observed in 70% of esophageal SCC and is associated with invasion, metastasis, and poor prognosis. Hypermethylation of *RAR*β and *RASSF1A* that causes cell cycle deregulation is found in 50% to 60% of esophageal SCC. As in SCC, esophageal adenocarcinoma is also characterized by frequent methylation of *APC*, *CDH1*, and $p16^{INK4a}$.[79] CDH1 methylation and reduced expression are associated with metastatic ability. Hypermethylation of $p14^{ARF}$ and $p15^{INK4b}$ is uncommon in Barrett esophagus–associated carcinogenesis. The lower frequency (10%) of hMLH1 methylation is consistent with the lower prevalence of MSI in esophageal adenocarcinoma compared with gastric and colorectal adenocarcinomas. *MGMT* inactivation by DNA methylation is frequently found in Barrett esophagus (40%) and esophageal adenocarcinoma (60%), whereas 20% of normal squamous epithelia show *MGMT* methylation.

The aberrant methylations mentioned above can be used as biomarkers for the molecular diagnosis of esophageal cancer. Methylation profiles of multiple genes including *APC*, *CDH1*, *MGMT*, *p16*, and *RUNX3* serve as indicators of the neoplastic progression of Barrett esophagus and are independent prognostic factors for esophageal adenocarcinoma.[81,82] DNA methylation detected in serum is useful for screening and monitoring of esophageal cancers. P16 methylation is found in the sera of 10% to 20% of esophageal SCC patients and correlates with poor prognosis.[83-85] APC methylation is observed in the sera of 25% of esophageal adenocarcinoma patients and in the sera of <10% of esophageal SCC patients, and high serum levels of APC methylation are significantly associated with reduced patient survival.[86]

### Molecular Diagnosis of Micrometastasis and CTCs

Sentinel node mapping for esophageal cancer is relatively complicated compared with that for gastric cancer, but it provides useful information on individualized selective lymphadenectomy, which reduces morbidity and maintains the quality of life for esophageal cancer patients.[87] Detection rates of sentinel nodes by the $^{99m}$Tc–tin colloid method or fluorescent dye imaging have

been reported to be satisfactory; the sensitivity is 90% to 100% for clinical stage T1 to T3 patients and 45% for patients who received neoadjuvant chemoradiation therapy, respectively.[38,89] A sensitive real-time RT-PCR system, using CK19, CK20, SCC antigen, and CEA as marker mRNAs, efficiently detects micrometastasis in sentinel nodes. CTCs in the blood can also be detected in esophageal cancer patients by the RT-PCR–based molecular method. CEA mRNA is detected in the sera of 60% of esophageal SCC patients and is correlated with tumor invasion, vessel involvement, nodal metastasis, and advanced stage.[90,91] The presence of CTCs detected by CEA expression is an independent factor for a shortened hematogenous disease-free interval. CTC positivity is correlated with reduced E-cadherin expression in the primary tumor. Detection of CTC by the RT-PCR–based method is useful for predicting recurrence in patients with esophageal SCC.

## miRNA-Based Molecular Diagnosis

Altered expression pattern of miRNAs has potential clinical applications toward developing biomarkers to identify the presence and progression of esophageal cancer and to assess tumor chemosensitivity and radiosensitivity.[92,93] In esophageal SCC, expressions of miR-10b, miR-92a, miR-93, miR-192, miR-194, and miR-205 are increased in tumor tissues compared with normal esophageal mucosa, while expressions of miR-100, miR-125b, miR-133a, miR-133b, miR-143, miR-145, miR-203, miR-205, and miR-375 are reduced. Overexpression of miR-21, miR-23a, miR-26a, miR-96, miR-103, miR-107, miR-128b, and miR-129 detected in esophageal SCC is correlated with prognosis. Increased expression of miR-200c correlates not only with poor prognosis but also with diminished sensitivity to chemotherapy in patients with esophageal SCC. Increased expression of miR-296 is also associated with chemoresistance. Detection of circulating miRNAs provides a new complementary tumor marker for esophageal SCC. The plasma level of miR-21 is higher and that of miR-375 is lower in SCC patients than in controls.[94] High plasma concentrations of miR-21 show significant correlation with recurrence.

On the other hand, increased expression of miR-21, miR-192, miR-195, and miR-223 and reduced expression of miR-203 are found in esophageal adenocarcinoma.[93] The levels of miR-30e and miR-200a correlate with survival of esophageal adenocarcinoma patients, whereas reduced miR-375 expression is associated with shorter survival.[95] Overexpression of miR-148 enhances the effect of cisplatin and 5-FU, providing a basis for the potential use of miRNAs to predict or improve the response to chemotherapy.[96] During the progression from normal mucosa to adenocarcinoma via Barrett esophagus, sequential upregulation of miR-21, miR-93, miR-192, and miR-194 is observed.[97] Upregulation of miR-192 and miR-215 and downregulation of miR-203, miR-205, and let7c are a "progression signature" for the progression from Barrett esophagus to adenocarcinoma and may serve as molecular markers for neoplastic progression.[98]

## Genetic Polymorphism and Esophageal Cancer Risk

Many studies have evaluated genetic polymorphism and esophageal cancer risk, with a majority of these studies conducted in Asian countries.[99-101] Meta-analyses of ALDH2, MTHFR, CYP1A1, CYP2E1, GSTP1, GSTM1, and GSTT1 have found significant correlations between ALDH2*1*2 and CYP1A1 Val allele and increased risk of esophageal cancer. *ALDH2* is a polymorphic gene, and individual genotypes determine blood concentrations of acetaldehyde after drinking, whereas *CYP1A1* is involved in the activation of major classes of tobacco procarcinogens such as polyaromatic hydrocarbons and aromatic amines. Increased risk of esophageal SCC is associated with ADH2*1*2 and p53 codon 72 Pro/Pro genotypes. GSTP1 (Ile105Val) is a risk factor for Barrett esophagus and adenocarcinoma in Caucasian males.[100] GSTP1 is the major isoform expressed in the esophagus and eliminates DNA oxidative products. In addition to protein-coding genes, pre-miRNAs also possess polymorphisms that affect esophageal cancer risk.[92] For instance, C–T SNP in pre-miR-196a (rs11614913) and G > C variant in pre-miR-146a increase esophageal SCC in the Chinese population.[92] These findings are applicable to molecular diagnosis in identifying individuals at high risk for developing esophageal cancer.

## Novel Molecular Markers Identified through SAGE Data Analysis

Although conventional serum tumor markers such as SCC antigen and CYFRA21-1 (fragment of CK19) have been used clinically as biomarkers, they have low sensitivity and low specificity. To search for novel biomarkers for esophageal cancer, a SAGE library was generated from esophageal SCC and compared with the library from normal esophageal mucosa.[102] Many upregulated and downregulated genes were identified that might be candidate diagnostic markers and therapeutic targets (Table 29.2). ADAM metalloproteinase with thrombospondin type 1 motif 16 (ADAMTS16) was the most upregulated gene in esophageal SCC. ADAMTS16 is expressed in 40% of esophageal SCC at high levels, as shown by quantitative RT-PCR, whereas SCCA1-encoding SCC antigen is expressed in only 20% of esophageal SCC. ADAMTS protein is secreted from cancer cells, and knockdown of ADAMTS16 inhibits cell growth and invasion ability. Thus, ADAMTS16 could be a novel diagnostic and therapeutic target in patients with esophageal SCC. SAGE data provide a list of genes associated with the development and progression of esophageal cancer.

**TABLE 29-2 The 10 Most Upregulated and Downregulated Tags/Genes in Esophageal Squamous Cell Carcinoma in Comparison with Normal Esophagus by Sage Data Analysis**

| | Tags per Million | | | |
|---|---|---|---|---|
| Upregulated Tag Sequence | SCC | Normal Esophagus | Symbol | Description |
| TCCCCTACAT | 2564[a] (37)[b] | 0 (0) | *ADAMTS16* | ADAM metallopeptidase with thrombospondin type 1 motif, 16 |
| GAAATAAAGC | 2495 (36) | 0 (0) | *IGHG1* | Immunoglobulin heavy constant gamma 1 (G1m marker) |
| TTCGGTTGGT | 2148 (31) | 0 (0) | *OGFOD1* | 2-Oxoglutarate and iron-dependent oxygenase domain containing 1 |
| AGGCATTGAA | 5336 (77) | 20 (1) | *NUTF2* | Nuclear transport factor 2 |
| CAGTTACAAA | 5544 (80) | 40 (2) | *RYBP* | RING1 and YY1 binding protein |
| TGGAAATGAC | 1317 (19) | 0 (0) | *COL1A1* | Collagen, type I, alpha 1 |
| ACCAAAAACC | 1663 (24) | 20 (1) | *COL1A1* | Collagen, type I, alpha 1 |
| GGCAGCACAA | 1455 (21) | 20 (1) | *NBEAL2* | Neurobeachin-like 2 |
| TTTATTAGAA | 1455 (21) | 20 (1) | *CCDC75* | Coiled-coil domain containing 75 |
| AGCCAAAAAA | 2980 (43) | 40 (2) | *MAP3K12* | Nuclear casein kinase and cyclin-dependent kinase substrate 1 |
| GTGGCCACGG | 0 (0) | 25283 (1277) | *S100A9* | S100 calcium binding protein A9 (calgranulin B) |
| GGCAGAGAAG | 0 (0) | 8454 (427) | *KRT4* | Keratin 4 |
| ATGAGCTGAC | 0 (0) | 3762 (190) | *CSTB* | Cystatin B (stefin B) |
| | | | *XPO7* | Exportin 7 |
| GAAGCACAAG | 0 (0) | 2475 (125) | *KRT6C* | Keratin 6C |
| TAATTTGCAT | 0 (0) | 2455 (124) | *EMP1* | Epithelial membrane protein 1 |
| | | | *GNA13* | Guanine nucleotide binding protein (G protein), alpha 13 |
| AAAGCGGGGC | 0 (0) | 2356 (119) | *KRT13* | Keratin 13 |
| TGTGTTGAGA | 0 (0) | 2257 (114) | *EEF1A1* | Eukaryotic translation elongation factor 1 alpha 1 |
| CACAAACGGT | 0 (0) | 2079 (105) | *TSPAN9* | Tetraspanin 9 |
| | | | *RPS27* | Ribosomal protein S27 |
| TGGTGTTGAG | 0 (0) | 1841 (93) | *RPS18* | Ribosomal protein S18 |
| GCCAATCCAG | 0 (0) | 1802 (91) | *CRNN* | Cornulin |

[a]The absolute tag counts are normalized to 1,000,000 total tags/sample.

[b]Number in parentheses indicates the absolute tag counts.

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# Cytokeratin Expression Profiling in Gastric Carcinoma: Clinicopathologic Significance and Comparison with Tumor-Associated Molecules

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**Key Words**
Gastric cancer · Cytokeratin · Mucin phenotype · Tumor-associated molecules

**Abstract**
***Objective:*** The expressions of cytokeratin (CK) 7 and 20 have been studied in various primary and metastatic carcinomas, and their determination may help distinguish the site of origin of metastatic carcinomas. However, little is known about the molecular basis that determines variations in CK patterns in gastric cancers (GCs). The aim of the present study was to analyze the CK expression patterns in a large number of GCs and to investigate how the CK patterns correlate with clinicopathologic parameters, histology, mucin phenotype or several tumor-related molecules. ***Methods and Results:*** We immunohistochemically examined the CK7/CK20 patterns, mucin expression profiles (MUC5AC, MUC6, MUC2 and CD10), and the cancer-related molecules (CDX2, p53, EGFR and β-catenin), using a tissue microarray with 870 GCs. The GCs were divided into four patterns; 17% of CK7+/CK20+, 57% of CK7+/CK20–, 9% of CK7–/CK20+ and 17% of CK7–/CK20. GCs with the CK7–/CK20– pattern demonstrated a close relation to undifferentiated adenocarcinoma. CK7 expression was significantly correlated with the expression of MUC5AC and MUC6, while CK20 expression was correlated with MUC2 and CDX2. There were statistically significant associations between CK expression patterns and mucin phenotypes. ***Conclusion:*** These results indicate that the CK7/CK20 expression patterns in GCs demonstrated different clinicopathologic features and molecular signatures.


## Introduction

Gastric cancer (GC) is one of the most common malignancies worldwide and often metastasizes to other organs, including the liver, lung and ovary [1]. In metastatic carcinomas of unknown primary site, identification of the origin as the stomach or other primary site is very difficult because of the heterogeneous histology of GC. This heterogeneity may be partly due to the fact that GC is caused by exogenous (nitrosamines and *Helicobacter pylori*) and endogenous (E-cadherin mutation) factors [2]. To identify these phenotypical differences at a morphologic level in a comparable and reproducible manner, it is

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necessary to have a novel classification system that recognizes all types of GCs adequately. In addition to classification by histology (the Lauren classification, the Japanese Classification of Gastric Carcinoma, etc.), GCs may also be classified into four phenotypes by the mucin expression profile: G type (gastric phenotype), I type (intestinal phenotype), GI type (gastric and intestinal mixed phenotype) and N type (neither gastric nor intestinal phenotype) [2, 3]. The G type is considered to behave more aggressively than the I type. Mutations of *p53* and loss of heterozygosity of the adenomatous polyposis coli gene occur more frequently in the I type than in the G type, while microsatellite instability and alterations in the *p73* gene are more common in the G type than in the I type. Microsatellite instability in the G type is usually associated with inactivation of hMLH1 following the promoter hypermethylation. The caudal-related homeobox gene 2 (CDX2) acts as an intestine-specific transcription factor and is expressed in I type GCs at high levels. CDX2 upregulates the expression of goblet-specific MUC2 [4].

Cytokeratin (CK), an intermediate filament observed mainly in epithelial cells, is an important cytoskeletal component involved in fixation of the nucleus and maintenance of cell morphology. CK consists of 20 subtypes, whose expression depends primarily on the epithelial cell type and the degree of differentiation [5]. The expression of CK8, CK18 and CK19 is observed at all levels of gastric mucosa, but CK20 expression is limited to mature superficial foveolar epithelium [6–8]. CK7 expression is absent in normal gastric mucosa but is observed in chronic mucosal irritation conditions such as *H. pylori* gastritis in the basal localization [9]. There are many reports of CK expression in various organs and the findings suggest that the CK expression profiles of metastatic cancers correspond to those of the primary sites [10–12]. In particular, in the gastrointestinal tract, colorectal carcinomas demonstrate a CK7–/CK20+ expression pattern, whereas adenocarcinomas of foregut origin demonstrate a CK7+/CK20– expression pattern [10–13]. Profiles for CK expression patterns may be helpful; however, the total number of GCs in each previous report about the expression of CK subtypes is one hundred cases at most [5, 6, 9, 12–21].

The aim of the present study was to analyze the CK expression patterns in a large number of GCs and to investigate how the CK expression patterns correlate with clinicopathologic parameters, histology, mucin phenotypes or several tumor-related molecules. Because the functional and biological properties of the GCs may reflect the tumors' ability to produce certain CKs, it would be of interest to determine which factors are best correlated with the CK immunophenotype.

## Materials and Methods

*Tissue Samples and Tissue Microarray Construction*

The surgical pathology files of the Hiroshima University Hospital and its affiliated hospitals were used to randomly select 870 GCs from 51 adenocarcinomas of the esophagogastric junction (AEG) and 819 distal GCs (corpus or antrum). Surgically resected specimens were routinely fixed in 10% buffered formalin and examined macroscopically. Tumor staging was performed according to the Union Internationale Contre le Cancer (UICC) system [22]. There were 403 T1, 257 T2, 184 T3 and 26 T4 in these 870 cases. Nodal metastasis was present in 366 patients (42%). Tumor staging revealed 500 stage I, 152 stage II, 154 stage III and 64 stage IV. GCs were histologically classified as 507 intestinal type and 363 diffuse type according to the Lauren classification system. The 507 intestinal type GCs included 63 papillary, 173 well-differentiated tubular and 271 moderately differentiated tubular adenocarcinomas. The 363 diffuse type GCs consisted of 297 poorly differentiated adenocarcinomas, 52 signet-ring cell carcinomas and 14 mucinous adenocarcinomas according to the WHO histological classification. In addition, the GCs were classified as 63 papillary (pap), 173 well differentiated tubular (tub1), 271 moderately differentiated tubular (tub2), 75 solid type poorly differentiated (por1), 222 non-solid type poorly differentiated (por2) adenocarcinomas and 52 signet-ring cell carcinoma (sig) and 14 mucinous adenocarcinomas (muc) according to the Japanese Classification of Gastric Carcinoma [23]. In accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government, tissue specimens were collected and used after approval from the Ethical Review Committee of the Hiroshima University School of Medicine and from the ethical review committees of collaborating organizations.

The two most representative portions to be sampled for the tissue microarrays (TMAs) were carefully selected from different intratumoral areas in each case and marked on the HE-stained slide. Two superficial areas in mucosal GCs, and one superficial area and one deep area in GCs that had invaded beyond the submucosa were selected. A 2-mm-diameter tissue core of each donor block was punched out and transferred to a recipient block with a maximum of 48 cores using a tissue microarrayer (AZUMAYA KIN-1, Tokyo, Japan). 5-μm-thick sections were cut from the recipient block and transferred to glass slides. HE staining was performed on TMA for confirmation of the tumor tissue. Each tissue-array block contained 21 cases of GC and four cases of non-neoplastic stomach samples.

*Immunohistochemistry*

A Dako Envision Kit (Dako, Carpinteria, Calif., USA) was used for immunohistochemical analysis of all markers. In brief, sections were pretreated by microwaving (500 W) in a citrate buffer (pH 6.0) for 15 min to retrieve antigenicity. After endogenous peroxidase activity was blocked with 3% $H_2O_2$-methanol for 10 min, sections were incubated with normal goat serum (Dako) for

**Table 1.** Antibodies used in the current study and their positive rates in 870 GCs

| Antibody | Clone | Dilution | Source | Positive cases n (%) |
|---|---|---|---|---|
| CK7 | OV-TL 12/30 | 1:50 | DAKO, Carpinteria, Calif., USA | 648 (74) |
| CK20 | IT-Ks 20.8 | 1:50 | DAKO, Carpinteria, Calif., USA | 232 (27) |
| MUC5AC | CLH2 | 1:50 | Novocastra, Newcastle, UK | 519 (60) |
| MUC6 | CLH5 | 1:50 | Novocastra, Newcastle, UK | 233 (27) |
| MUC2 | Ccp58 | 1:50 | Novocastra, Newcastle, UK | 199 (23) |
| CD10 | 56C6 | 1:50 | Novocastra, Newcastle, UK | 71 (8) |
| p53 | DO-7 | 1:50 | Novocastra, Newcastle, UK | 293 (34) |
| EGFR | EGFR.113 | 1:50 | Novocastra, Newcastle, UK | 109 (13) |
| CDX2 | AMT28 | 1:20 | BioGenex, San Ramon, Calif., USA | 183 (21) |
| β-Catenin | 14/β-catenin | 1:50 | BD Biosciences, San Jose, Calif., USA | 140 (16) |

The cutoff point for antibody reactivity necessary to define a result as positive was staining of at least 10% of cancer cells in the TMAs.

20 min to block nonspecific antibody binding sites. Sections were then incubated with the following primary antibodies: anti-CK7, anti-CK20, anti-MUC5AC, anti-MUC6, anti-MUC2, anti-CD10, anti-p53, anti-EGFR, anti-CDX2 and anti-β-catenin. Suppliers and working dilutions are noted in table 1. Sections were incubated with a primary antibody for 1 h at 25°C, followed by incubations with peroxidase-labeled anti-rabbit or mouse IgG for 60 min. Staining was completed with a 10-min incubation with the substrate-chromogen solution. The sections were counterstained with 0.1% hematoxylin. Appropriate positive and negative control samples were used.

*Evaluation of Positive Cases and Cutoff-Point Thresholds*

Immunostaining results were evaluated independently by 3 investigators (H.T., K.S. and M.M.) and when the evaluations differed, a decision was made by consensus while the investigators reviewed the specimen with a multihead microscope. Neoplastic tissue was evaluated semiquantitatively at magnifications of ×100 and ×400. Cytoplasmic immunoreactivity for CK7, CK20, MUC5AC, MUC6 and MUC2, membranous reactivity for CD10 and EGFR, and nuclear reactivity for p53, CDX2 and β-catenin were assessed (fig. 1). The cutoff point for antibody reactivity necessary to define a result as positive was staining of more than 10% tumor cells in the TMAs.

*CK Expression Profiles and Mucin Phenotypes*

The 870 GCs were evaluated according to the CK7 and CK20 staining pattern and classified into four main groups: (1) coexpression of CK7 and CK20 (CK7+/CK20+), (2) no expression of CK7 and CK20 (CK7–/CK20–), (3) only CK7 expression (CK7+/CK20–) and (4) only CK20 expression (CK7–/CK20+). The criteria [24] for classification of G type and I type were as follows: GCs in which more than 10% of the cells displayed the gastric (MUC5AC and/or MUC6) or intestinal epithelial cell phenotype (MUC2 and/or CD10) were G type or I type, respectively. Those sections that showed both G and I types were classified as GI type, and those that lacked both G and I types were classified as N type.

*Statistical Methods*

Associations between CK expression profiling and clinicopathologic variables, or immunostaining for various markers were analyzed by the $\chi^2$ test. $p < 0.001$ was considered statistically significant.

## Results

*CK Expression Patterns in GCs and Their Correlation with Clinicopathologic Parameters*

Immunohistochemical results in the current study are shown in table 1. The 870 GCs included 648 (74%) cases with CK7 expression and 232 (27%) cases with CK20 expression, and were classified into 156 (17%) cases with CK7+/CK20+ pattern, 492 (57%) cases with CK7+/CK20– pattern, 76 (9%) cases with CK7–/CK20+ pattern and 146 (17%) cases with CK7–/CK20– pattern. We investigated the relation between CK expression patterns and the clinicopathologic parameters including age, sex, tumor location, T grade, N grade, M grade, staging and histological type according to the Lauren classification. As shown in table 2, the CK7–/CK20– pattern was observed more frequently in the diffuse type of GC than in the intestinal type ($p = 0.0003$). In contrast, no differential trend was found between other CK expression patterns and clinicopathologic parameters. Regarding the Japanese Classification of Gastric Carcinomas, the CK7–/CK20– pattern was observed more frequently in the undifferentiated type (por1, por2, sig and muc) than in the differentiated type GC (pap, tub1 and tub2) (fig. 2; $p = 0.0003$). Furthermore, the analysis according to the WHO classification also yielded a similar result.

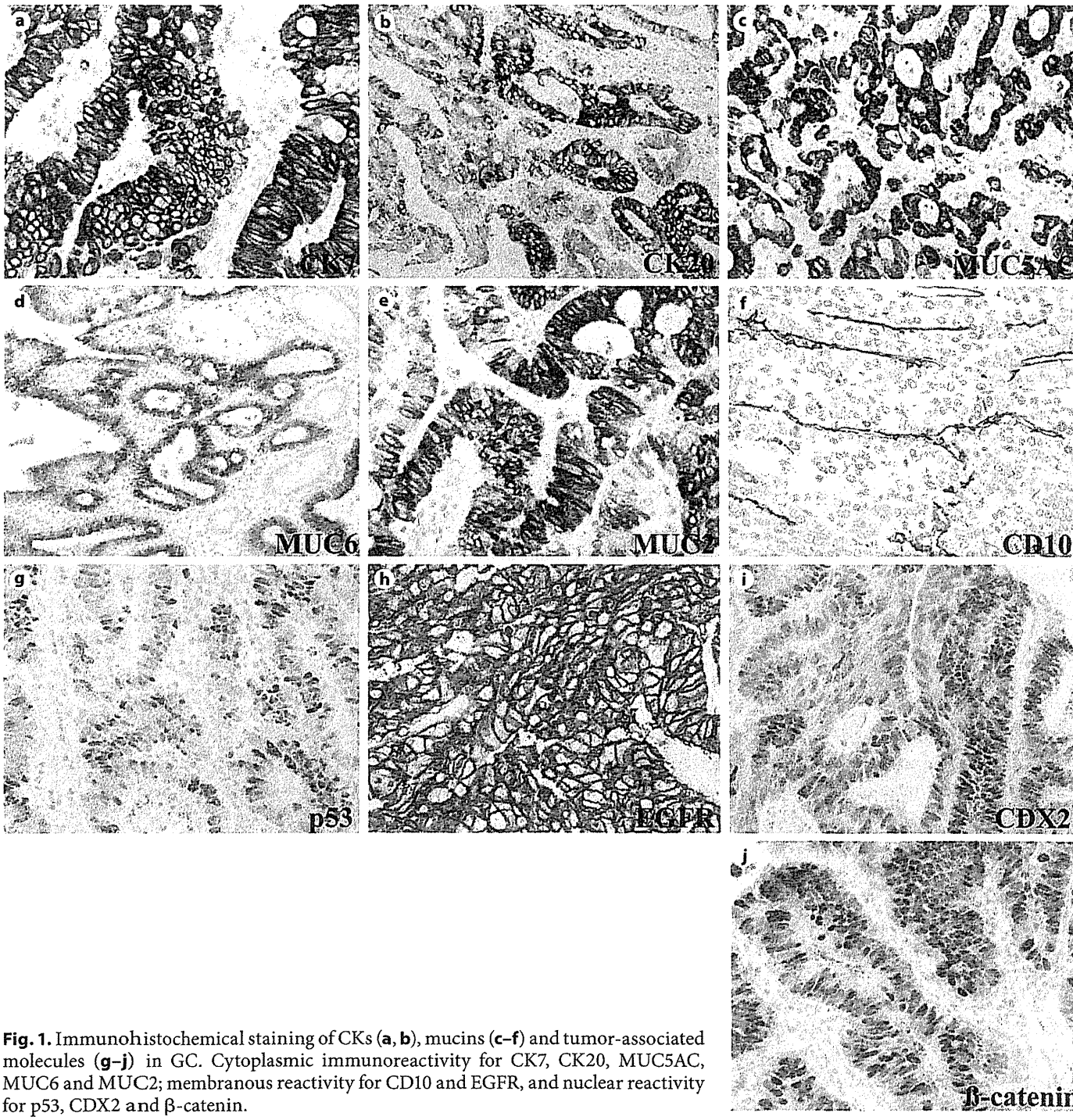


**Fig. 1.** Immunohistochemical staining of CKs (**a**, **b**), mucins (**c–f**) and tumor-associated molecules (**g–j**) in GC. Cytoplasmic immunoreactivity for CK7, CK20, MUC5AC, MUC6 and MUC2; membranous reactivity for CD10 and EGFR, and nuclear reactivity for p53, CDX2 and β-catenin.

*Distribution of Mucin Phenotypes and Histological Types of GC*

Next, we analyzed the relationships between the histological type and the mucin phenotype in the GCs. The 870 GCs included 519 (60%) cases with MUC5AC expression, 233 (27%) cases with MUC6 expression, 199 (23%) cases with MUC2 expression and 71 (8%) cases with CD10 expression. They were classified into 313 (36%) G type, 196 (23%) GI type, 149 (17%) I type and 212 (24%) N type. The distribution of each mucin phenotype and

**Table 2.** Relationships between CK expression patterns and clinicopathological findings in 870 GCs

| CK pattern | | CK7+/CK20+ (n = 156) | CK7+/CK20– (n = 492) | CK7–/CK20+ (n = 76) | CK7–/CK20– (n = 146) | p value |
|---|---|---|---|---|---|---|
| Age | >65 years | 74 (47) | 258 (52) | 35 (46) | 79 (54) | NS |
| | ≤65 years | 82 (53) | 234 (48) | 41 (54) | 67 (46) | |
| Sex | Male | 93 (60) | 322 (65) | 41 (54) | 97 (66) | NS |
| | Female | 63 (40) | 170 (35) | 35 (46) | 49 (34) | |
| Tumor location | AEG | 10 (6) | 28 (6) | 6 (8) | 7 (5) | NS |
| | Distal GC | 146 (94) | 464 (94) | 70 (92) | 139 (95) | |
| T grade | T1 | 91 (58) | 214 (43) | 29 (38) | 69 (47) | NS |
| | T2/T3/T4 | 65 (42) | 278 (57) | 47 (62) | 77 (53) | |
| N grade | N0 | 107 (69) | 279 (57) | 36 (47) | 82 (56) | NS |
| | N1 | 49 (31) | 213 (43) | 40 (53) | 64 (44) | |
| M grade | M0 | 155 (99) | 484 (98) | 76 (100) | 146 (100) | NS |
| | M1 | 1 (1) | 8 (2) | 0 | 0 | |
| Staging | Stage I | 106 (68) | 277 (56) | 36 (47) | 81 (55) | NS |
| | Stage II/III/IV | 50 (32) | 215 (44) | 40 (53) | 65 (45) | |
| Histology | Intestinal type | 105 (67) | 285 (58) | 52 (68) | 61 (42) | 0.0003 |
| | Diffuse type | 51 (33) | 207 (42) | 24 (32) | 85 (58) | |

Values in parentheses are percentages. AEG = Adenocarcinoma of the esophagogastric junction; NS = not significant. A $p < 0.001$ was considered statistically significant by $\chi^2$ test. Tumor staging was classified according to the criteria of the International Union Against Cancer TNM classification of malignant tumors. Histology was according to the Lauren classification system.

histological type according to the Japanese Classification of Gastric Carcinomas is shown in figure 2. The N type was observed more frequently in the undifferentiated type GC.

*Association of Expression between CK Patterns and Various Molecules*

We then investigated the association between CK expression patterns and various molecules in the GCs. Of the 870 GCs examined, each tumor-associated molecule was detected in 293 (34%) cases for p53, 109 (13%) cases for EGFR, 183 (21%) cases for CDX2 and 140 (16%) cases for β-catenin (table 1). There are statistically significant associations between CK7 and MUC5AC expression, CK7 and MUC6 expression, CK20 and MUC2 expression, and CK20 and CDX2 ($p < 0.0001$) (fig. 3).

*Association of CK Expression Patterns with Mucin Phenotypes*

The relationship between each CK expression pattern and mucin phenotype in the 870 GCs was analyzed. As shown in figure 4, there are statistically significant associations between CK7 expression without CK20 expression and G type, CK7 expression and GI type, CK20 expression without CK7 expression and I type, and neither of them and N type, respectively ($p < 0.0001$).

## Discussion

Much interest has focused on CK immunoprofiles in the classification of carcinomas, in particular the CK7 and CK20 profiles. There are many reports on the expression of CK7 and CK20 in relatively small numbers of GC cases [5, 6, 9, 12–21]. CK7 immunoreactivity is reported to range from 10 to 75%, while CK20 expression is frequently reported to range from 30 to 50%. Our results are also consistent with the findings of previous reports. The differences of positive rates in these previous reports are possibly due to evaluation scales and case groups. In this study, we used the TMA method to examine each molecule expression in the GCs. Although minute TMAs cannot ensure representative areas of donor specimen, we used 2-mm-diameter needles, which are large enough to evaluate the morphological appearance if representative regions are carefully selected with HE slides [25, 26]. In

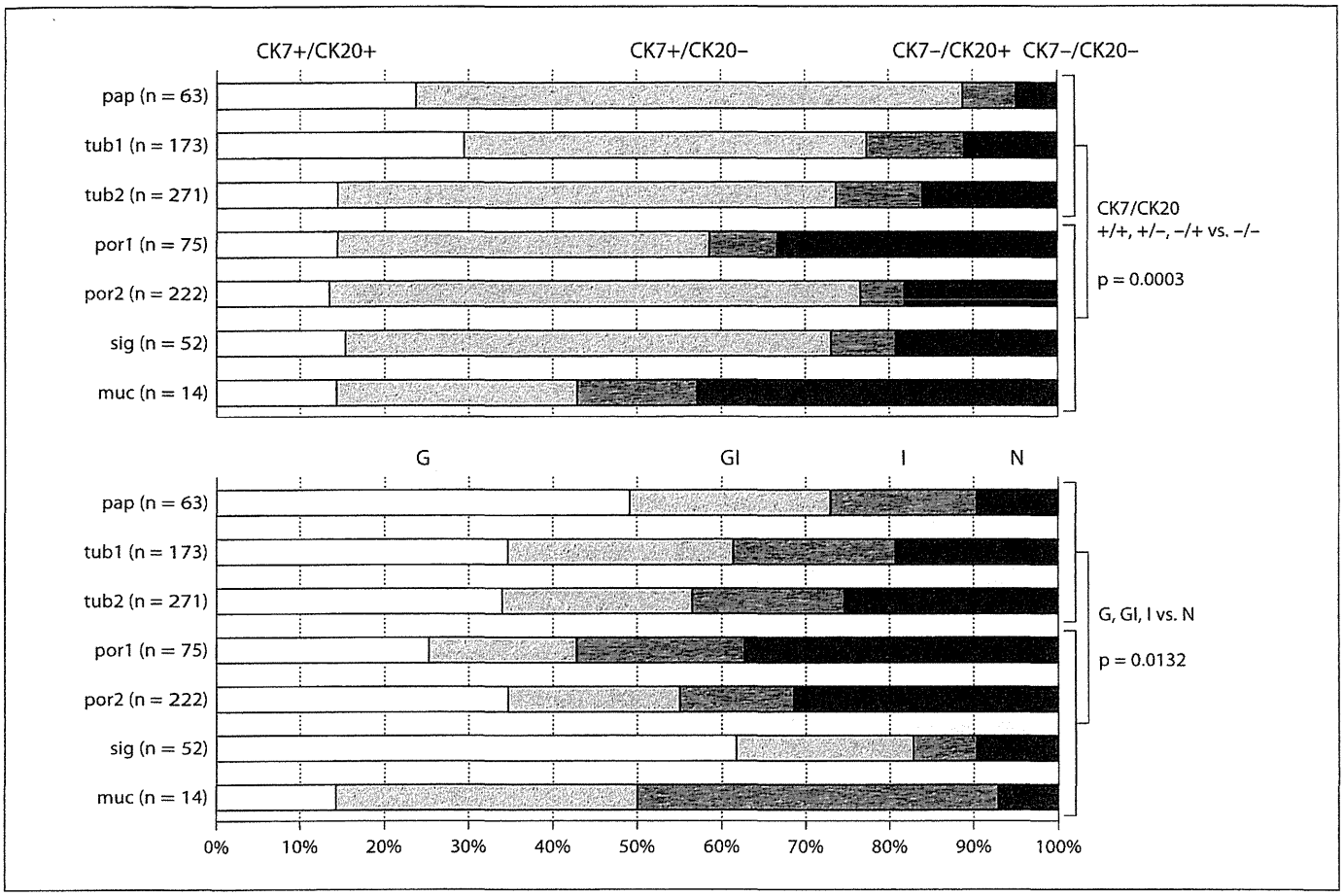


**Fig. 2.** The relationships between detailed histological type and CK expression patterns and mucin phenotypes in 870 GCs. The 870 GCs were histologically classified as 507 of the differentiated type (papillary adenocarcinoma or tubular adenocarcinoma) and 363 of the undifferentiated type (poorly differentiated adenocarcinoma, signet-ring cell carcinoma or mucinous adenocarcinoma). There are statistically significant associations between the undifferentiated type and neither CK7 nor CK20 expression, and the undifferentiated type and N mucin phenotype.

terms of the possible diversity of histological components or molecular abnormality in the GCs, several previous reports have shown an excellent concordance between the results obtained from TMAs and those from full sections [27, 28]. Furthermore, the effects of intratumoral heterogeneity can be averaged out in such a large-scale analysis as the present study. It is unlikely that the use of TMA biased the outcome.

To clarify the significance of the CK expression patterns, we analyzed the relation between CK expression patterns and the clinicopathologic parameters, histology, mucin phenotype or several tumor-related molecules. Histologically, expression of CK7 and/or CK20 showed a tendency toward a high positive rate in differentiated type GC and a low positive rate in undifferentiated type GC. This may reflect a loss of the ability to produce the CKs along with a decrease in histological differentiation in neoplastic cells. However, CK7 and/or CK20 expression was not associated with any other clinicopathologic features, consistent with the previous report [17, 29]. GCs have been classified into four mucin phenotypes. Previous reports provided evidence that mucin expression is closely associated with the differentiation of GCs [30, 31]. In the present study, G type was correlated with CK7 expression, especially in the absence of CK20 expression, whereas I type was correlated with CK20 expression, especially in the absence of CK7 expression. This result demonstrated statistical significance, but neither CK7 nor CK20 was sufficient for the discrimination of the mucin phenotype due to low sensitivity and specificity. In the present study,

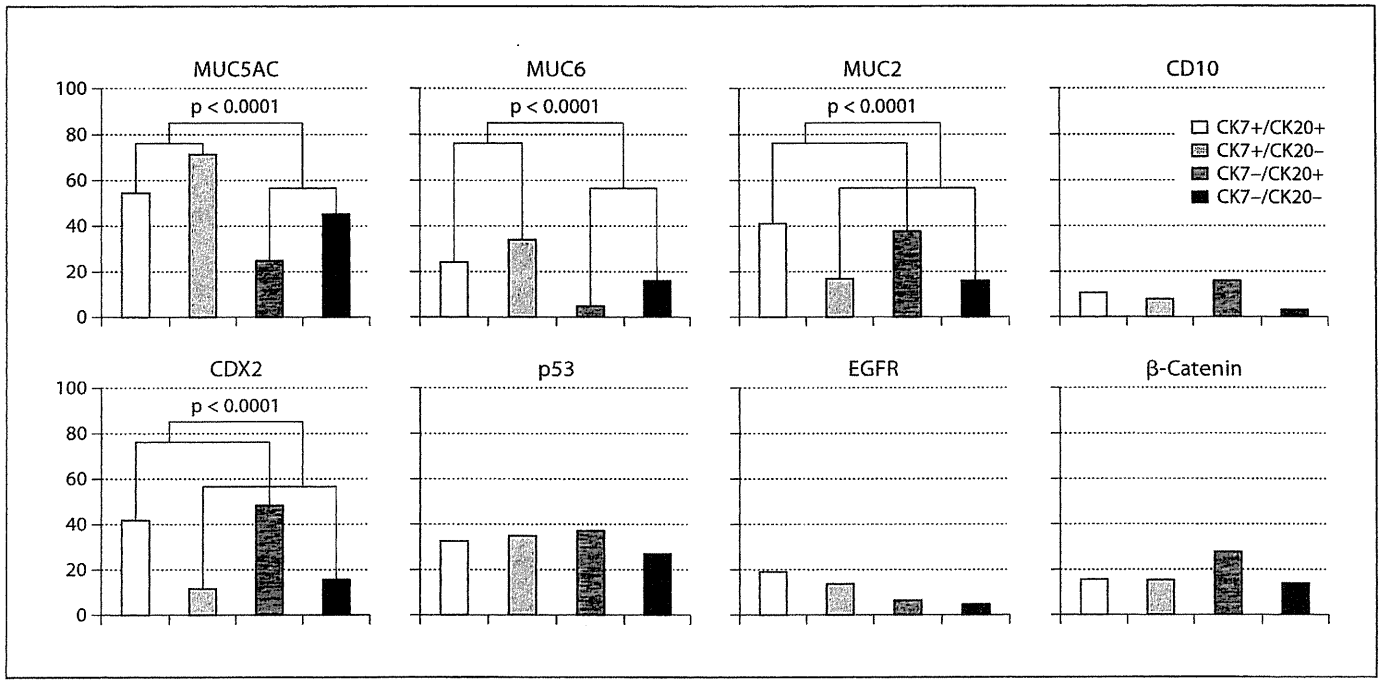


**Fig. 3.** The relationships between CK expression patterns and various markers in 870 GCs. There are statistically significant associations between CK7 and MUC5AC expression, CK7 and MUC6 expression, CK20 and MUC2 expression, and CK20 and CDX2 ($p < 0.0001$).

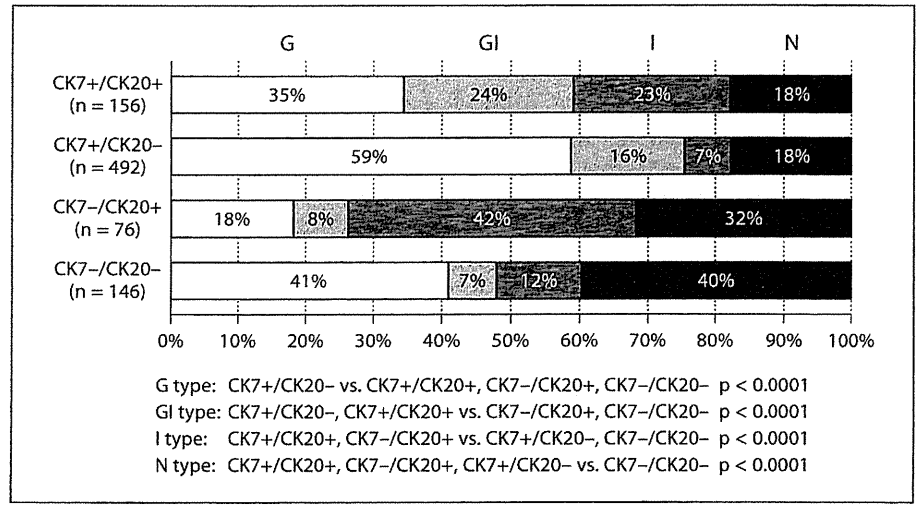


**Fig. 4.** The relationships between each CK expression pattern and mucin phenotypes in 870 GCs. There are statistically significant associations between CK7 expression without CK20 expression and G mucin phenotype, CK7 expression and GI mucin phenotype, CK20 expression without CK7 expression and I mucin phenotype, and neither of them and N mucin phenotype ($p < 0.0001$).

the positive expression of CK20 was frequently observed in GCs with the I type and showed significant correlation with the positive expression of Cdx2. There is no previous report showing a direct association between CK20 and Cdx2. Chan et al. [32] reported that CK20 is directly regulated by Cdx1. Therefore, there may also be a close correlation between CK20 and CDX2.

In summary, GCs showed heterogenous CK expression representing their histological features. Therefore, a single CK and its combination does not always provide diagnostic value in differentiating GCs. Advances in our understanding of the genetic and molecular bases of GC according to each CK expression, however, may lead to new therapy.

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Original Article

# Expression of cancer stem cell markers ALDH1, CD44 and CD133 in primary tumor and lymph node metastasis of gastric cancer

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**Gastric cancer (GC) is one of the most common malignancies worldwide. Recently, cancer stem cells (CSCs) in tumors were found to possess the ability to sustain tumor self-renewal, initiate tumor progression, and possibly also contribute to cancer metastasis. We immunohistochemically examined expression and distribution of representative CSC markers ALDH1, CD44, and CD133 in primary tumors and lymph node metastasis of GC. Among 190 GC primary tumors, 104 (55%) were positive for ALDH1, 117 (62%) were positive for CD44, and 18 (9%) were positive for CD133. Expression of these three CSC markers was significantly associated with advanced clinicopathologic factors. Patients with CD44- and CD133-positive GC had a poorer survival rate than patients with CD44- and CD133-negative GC (CD44: $P < 0.001$, CD133: $P = 0.006$). Univariate and multivariate Cox proportional hazards analysis revealed tumor node metastasis stage, CD44 expression, and CD133 expression to be independent predictors of survival in patients with GC. Comparison of CSC markers in primary and metastatic sites showed ALDH1 positivity to be significantly higher in diffuse-type lymph node metastasis than in the primary tumor ($P < 0.001$). These results indicate that these CSC markers are important in tumor invasion and metastasis and may be good markers indicating long-term survival in patients with GC.**

**Key words:** cancer stem cell, gastric cancer, metastasis, stem cell marker

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Gastric cancer (GC) is one of the most common human cancers. According to the World Health Organization, GC is the fourth most common malignancy worldwide, with approximately 870 000 new cases occurring yearly.[1] Cancer develops as a result of multiple genetic and epigenetic alterations. Cancer at an advanced stage is a systemic disease and metastatic status significantly affects patient outcomes. In GC, lymph node metastasis is one of the most valuable prognostic factors. It is an important issue to examine the biological characteristics of cancer cells not only in primary tumors but also in metastatic tumors in the lymph node. However, the molecular pathological features of metastatic sites have not been sufficiently analyzed.

Recently, the cancer stem cell model suggests that in many cancers, tumor initiation and propagation is driven by a population of self-renewing tumor cells known as cancer stem cells (CSCs).[2] CSCs also promote tumor cell heterogeneity, metastasis, and therapeutic resistance, and are potentially driven by known oncogenic signaling pathways.[3,4] The study of CSCs would be greatly enhanced by the availability of specific markers to identify and isolate these cells. Through examinations using putative stem cell markers or side population (SP), unique subsets of cancer cells from different types of tumors have been detected. These markers include CD133, CD44, CD24, and CD166. Among them, both CD133 and CD44 are widely used for isolating CSCs from solid tumors. CD133 is a cell surface transmembrane glycoprotein, which exists in the cholesterol-rich domain of lipid rafts, and was identified in subpopulations of cells in brain and colon tumors.[5,6] Only one hundred CD133-positive cells implanted in a non-obese diabetic severe combined immunodeficient (NOD-SCID) mouse are sufficient to initiate a tumor, and isolated stem cells from this tumor can be serially passed to other NOD-SCID mice.[5,7] Expression of CD133 has been reported to be associated with poor prognosis in

GC.[8,9] CD44 is also a transmembrane glycoprotein which participates in many cellular processes, including growth, survival, differentiation, and mortality,[10,11] and plays important roles in malignant behaviors of several human cancers including GC.[12–14] The CD44 marker can be used to isolate CSC populations of prostate,[15] pancreas,[16] and colorectal tumors.[17] Recent reports indicate that CD44-positive fractions of GC can generate spheroid colonies under non-adherent conditions and that small numbers of these cells can generate tumors in SCID mice.[18]

Beside these markers, a promising new marker for CSC is aldehyde dehydrogenase 1 (ALDH1).[19,20] Aldehyde dehydrogenase enzymes are a family of intracellular enzymes that participate in cellular detoxification, differentiation, and drug resistance through the oxidation of cellular aldehydes.[21] ALDH1 positive cells monitored by immunohistochemistry and flow cytometry occupy a considerably smaller subpopulation that is about one-seventh of the size of the CD44- and CD133-positive populations. Nevertheless, ALDH1-positive cell populations are capable of generating tumor xenografts.[20] Therefore, ALDH1 may be able to label a cell population closely related to stem cells. Moreover, high percentages of ALDH1-positive cells in most types of epithelial tumors are associated with poorer clinical outcomes for these patients.[19,22,23] However, expression of ALDH1 in GC has not been analyzed.

In this present study, we examined the expression and distribution of the representative CSC markers ALDH1, CD44 and CD133 in GC by immunohistochemistry, and studied their relationship with clinicopathologic features. Furthermore, the expression of CSC markers was compared between GC primary tumors and metastatic lymph nodes.

## MATERIALS AND METHODS

### Tissue samples

In total, 190 primary tumor samples were collected from patients diagnosed with GC. Patients were treated at the Hiroshima University Hospital or affiliated hospitals. Of the 190 GC samples, associated lymph node metastasis samples were available for 104 cases. Information on patient prognosis was available for 96 of the 190 GC cases. Because written informed consent was not obtained, for strict privacy protection, identifying information for all samples was removed before analysis. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government.

For immunohistochemical analysis, we used formalin-fixed, paraffin-embedded tissues from 190 patients who had undergone surgical excision for GC. One or two representative tumor blocks were examined from each patient by immunohistochemistry. Tumor staging was made according to the TNM classification system. Histological classification of GC was carried out according to the Lauren classification system; GC cases were classified into intestinal-, diffuse-, and mixed-type.

### Immunohistochemical examination

Immunohistochemical staining was carried out according to the procedure previously reported.[24] Deparaffinized sections were deparaffinized in xylene, dehydrated in a graded ethanol series, and immersed in a 0.3% hydrogen peroxide solution in methanol for 10 min to inhibit endogenous peroxidase activity. The sections were placed in ethylenediaminetetraacetic acid buffer at pH 8.0 for CD133, or citrate buffer (pH 6.0) for ALDH1 and CD44 stainings. For antigen retrieval, the slides were heated at 95°C for 20 min in a microwave oven for CD133, or 95°C for 30 min in a microwave oven for ALDH1 and CD44 staining, and allowed to cool for 5 min at room temperature. Sections were incubated with the following antibody dilutions; primary anti-CD133 antibody (AC133; Miltenyi Biotec, Auburn, CA, USA) 1:100, anti-ALDH1 antibody (BD Biosciences; San Diego, CA, USA) 1:200, and anti-CD44 antibody (Novocastra; Newcastle, UK). Sections were incubated with primary antibody for 1 h at room temperature. The slides were washed three times with PBS, followed by incubations with Envision+ anti-mouse peroxidase for 1 h. For color reactions, sections were incubated with the DAB Substrate-Chromogen Solution (Dako Cytomation; Carpinteria, CA, USA) for 10 min. Sections were then counterstained with 0.1% hematoxylin. Previous reports demonstrated that significant correlation between poor clinical outcome and immunostaining with CD133 in colorectal cancer,[25] CD44 in squamous cell carcinoma of lung,[26] and ALDH1 in non-small cell carcinoma of lung.[27] According to the definition of positive staining in these previous reports, the result was considered positive if at least 10% of the cells were stained. When fewer than 10% of cancer cells were stained, the immunostaining was considered negative.

### Statistical analysis

ALDH1, CD44, and CD133 expression and clinicopathologic features were tested for association by the $\chi^2$ test. For each molecule, Kaplan–Meier survival curves were constructed to compare positive and negative patients. Differences between survival curves were tested for statistical significance by log-rank test. Univariate and multivariate Cox regression was used to evaluate the associations between clinical covariates and cancer-specific mortality in SPSS (SPSS Inc., Chicago, IL, USA). Hazard ratio (HR) and 95% confidence interval (CI)