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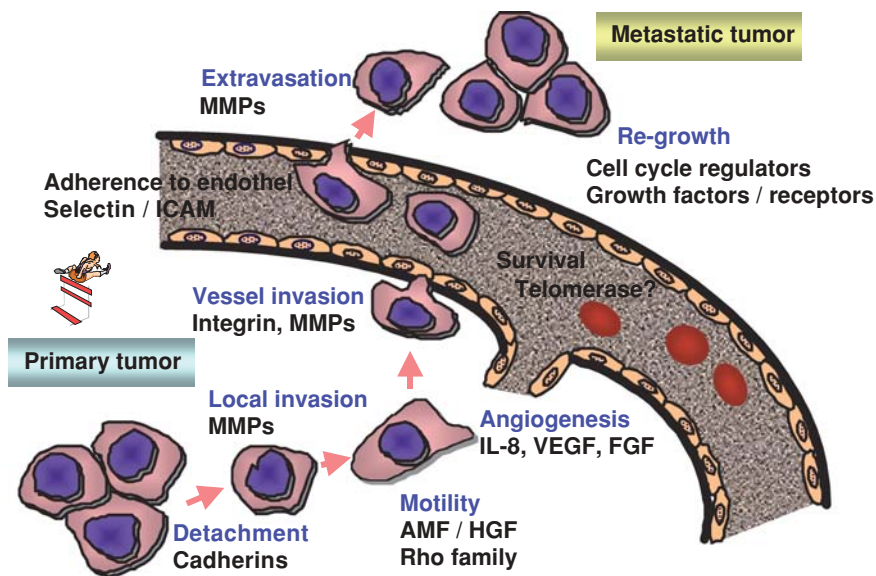
Fig. 1. Molecular mechanism of hematogenous metastasis. *MMPs*, matrix metalloproteinases; *IL*, interleukin; *VEGF*, vascular endothelial growth factor; *FGF*, fibroblast growth factor; *AMF*, autocrine motility factor; *HGF*, hepatocyte growth factor; *ICAM*, intercellular adhesion molecule

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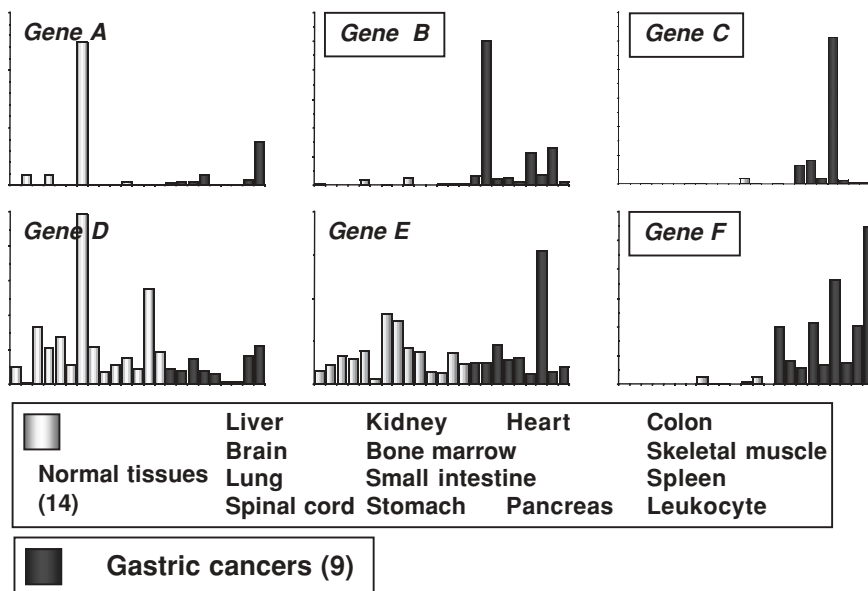
Fig. 2. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) of various normal tissues and gastric cancers. The expression of genes *B*, *C*, and *F* is confined to gastric cancers, while that of genes *A*, *D*, and *E* is detected in both normal tissues and gastric cancers at various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

ferentially 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
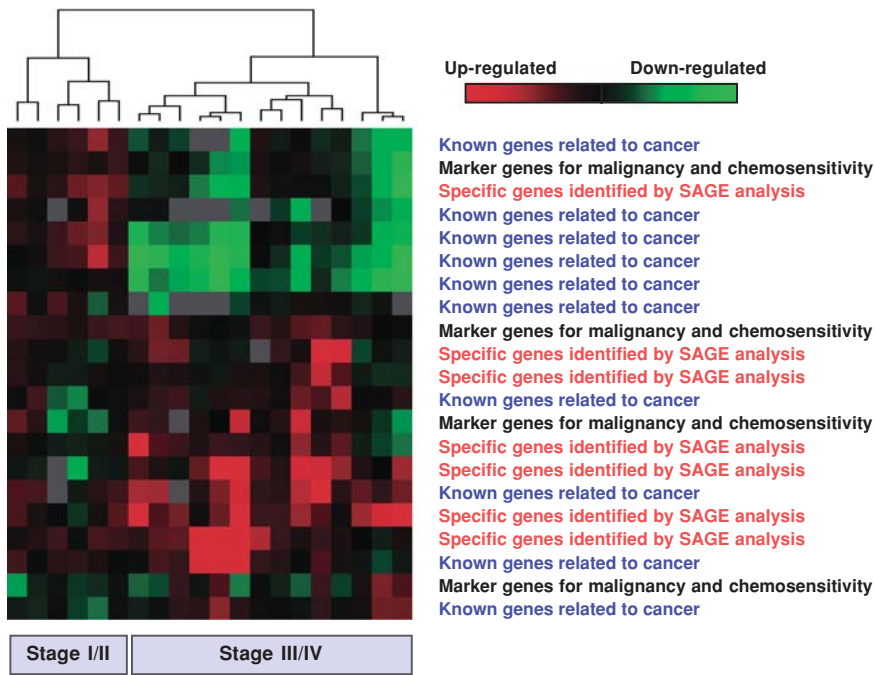
Fig. 3. Hierarchical clustering analysis of gene expression profiles by tumor stage. Gene expression profiles were examined in 20 surgically resected gastric cancer tissues after T7-based RNA amplification, using a mixture of normal gastric mucosal tissues as a reference. Twenty-one genes showing significant 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
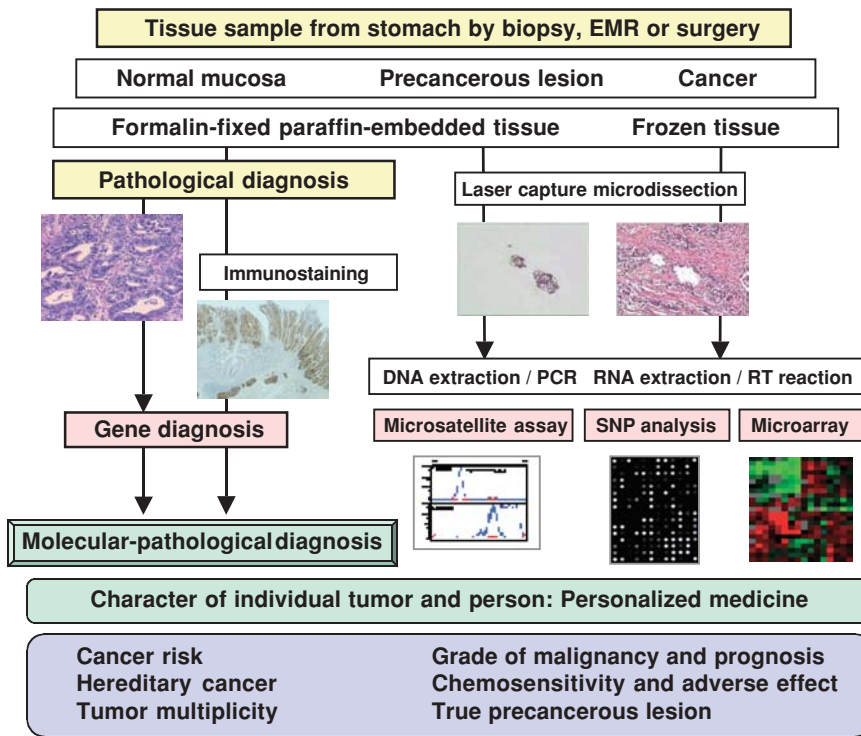

Fig. 4. Strategy for the molecular-pathological diagnosis of gastric cancer using tissue samples. *EMR*, endoscopic mucosal resection; *SNP*, single-nucleotide polymorphism

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 expres-sion 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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