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Prediction of peritoneal metastasis in advanced gastric cancer by gene expression profiling of the primary site

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ABSTRACT

Peritoneal metastasis is the most common cause of tumour progression in advanced gastric cancer. Clinicopathological findings including cytologic examination of peritoneal lavage have been applied to assess the risk of peritoneal metastasis, but are sometimes inadequate for predicting peritoneal metastasis in individuals. Hence, we tried to construct a new prediction system for peritoneal metastasis by using a PCR-based high throughput array with 2304 genes. The prediction system, constructed from the learning set comprised of 30 patients with the most informative 18 genes, classified each case into a 'good signature group' or 'poor signature group'. Then, we confirmed the predictive performance in an additional validation set comprised of 24 patients, and the prediction accuracy for peritoneal metastasis was 75%. Kaplan–Meier analysis with peritoneal metastasis revealed significant difference between these two groups ($P = 0.0225$). By combining our system with conventional clinicopathological factors, we can identify high risk cases for peritoneal metastasis more accurately.

1. Introduction

Gastric cancer, though its incidence is declining, remains a major cause of cancer death in the world.[1] Recent progress in diagnostic and treatment technologies has improved the long-term survival of patients with early gastric cancer, although the prognosis of patients with advanced gastric cancer remains unfavorable. Peritoneal metastasis is the most common cause of tumour progression in advanced gastric cancer.[2,3] Patients with identical clinical or pathological stages may differ widely in the clinical evolution. Clinicopathological findings including cytologic examination of peritoneal lavages have been applied to assess the risk of peritoneal metastasis, but are sometimes inadequate for predicting peritoneal metastasis in individuals. It is therefore important to identify high-risk patients for peritoneal metastasis.

Multiple genetic alterations are involved in the development and progression of gastric cancer,[4] and these aberrations may affect the expression of large number of genes.[5] Several molecules have been implicated in the peritoneal metastasis of gastric cancer.[6] However, it is clear that a single or even a few selected molecules will not define the whole biological characteristics of a tumour. Hence systematic

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analysis of gene expression profiling might be beneficial for understanding the biological potential of a tumour and useful for identifying high-risk patients for peritoneal metastasis.

Gene expression profiling has provided great insights into medical science. In fact, this approach has already been used to identify genes that could serve as molecular markers of cancer classification and outcome prediction.[7–9] Reverse transcriptase-polymerase chain reaction (RT-PCR) is a sensitive and reliable technology for analysis of gene expression. We therefore prepared a novel technology, a PCR-array technique, which is based on a high throughput quantitative RT-PCR, adaptor-tagged competitive PCR (ATAC-PCR).[10] This technique may be less complicated than DNA microarrays, which require thousands of spotted genes, and is advantageous in the manner of data treatment because this technique is based on a more popular technology, PCR. The reliability of this technique for cancer research has been established in previous work on gastric, colorectal, hepatocellular and breast carcinoma.[11–14] In this report, we constructed a prediction system for peritoneal metastasis in individual patients with advanced gastric cancer from the gene expression profiling analysis of 30 patients. The usefulness of this system for future clinical application was validated in additional 24 patients.

2. Materials and methods

2.1. Patients and clinical samples

We obtained advanced gastric cancer samples from 54 patients who underwent gastric resection at Osaka University Hospital. All tumours infiltrated into the subserosa or deeper on histopathological examination. None of the patients received chemotherapy or radiotherapy before surgery. Macroscopic and microscopic evaluations were made according to the general rules for gastric cancer study in surgery and pathology in Japan.[15] Follow-up was performed every three months for the first two years and every three to six months thereafter. Peritoneal recurrence was diagnosed by clinical and radiological examination or by reoperation. We assigned 30 cases (17 recurrence-free and 13 synchronous peritoneal metastasis cases) to the learning set and the remaining 24 cases (11 recurrence-free and 13 metachronous peritoneal metastasis cases) to the validation set, respectively. Recurrence-free cases were free of recurrence for at least three years after curative resection and all negative in cytologic examination of peritoneal lavages. We constructed a prediction system for peritoneal metastasis using the learning set and evaluated the predictive performance of this system using the validation set. All aspects of our study protocol were performed according to the ethical guidelines set by the committee of the three Ministries of the Japanese Government and a signed consent form was obtained from each subject.

2.2. PCR-array technique

Total RNA was purified from clinical samples utilizing TRIzol reagent (GibcoBRL, Grand Island, New York, USA). To select only genes specifically expressed in gastric tissues, we constructed two 3′-end-directed cDNA libraries from gastric cancer and normal gastric mucosa, as previously described.[16] Total RNAs were prepared from five gastric cancer tissues for the gastric cancer library, and three normal gastric mucosae for the normal gastric library. A total of 6525 EST clones were sequenced and we designed PCR primers for the ATAC-PCR reaction for a total of 1999 clones based on these results. Furthermore, we added 305 genes that had been reported in literatures to be involved in carcinogenesis or development of gastric cancer. Consequently, we prepared, in total, 2304 gene-specific primers for ATAC-PCR assay. The specificity of this gene set provides an advantage over more universal sets, which include genes not detected in gastric tissues.

ATAC-PCR is an advanced version of quantitative competitive PCR, characterised by using the 3′-end of mRNA and the addition of unique adaptors for different cDNAs. The ATAC-PCR experimental procedure was performed as previously described.[16] An equal amount of each RNA was applied for the conversion of mRNA to double-stranded cDNA using an oligo-(dT) primer and subsequent treatment with restriction enzyme *Mbo*I. Each target cDNA sample was ligated to one of the seven adaptors that share a common sequence outside, but each has a spacer region of different length. For an internal control of PCR, a standard cDNA sample was prepared from a mixture of all gastric cancer tissues used in this study. The standard sample treated with *Mbo*I was serially diluted to two concentrations, and each was ligated to another adaptor. The five target and two standard samples in the ligation mixtures were mixed and subjected to PCR in a reaction mixture containing an adaptor primer labeled with a fluorescent dye and a gene-specific primer. Each reaction mixture contained eight portions of standard sample with the shortest adaptor, two portions of that with the second shortest adaptor, and five portions of each target sample. The sequence of the adaptor-primer is the same as that of the common region of the adaptor. Amplified PCR products were separated using an ABI 3730 DNA analyzer. The detailed protocol is also available at our web site (http://genome.mc.pref.osaka.jp/ATACPCR.html). We performed ATAC-PCR assay with 2304 genes in all 54 tumours.

2.3. Data analysis

The relative expression of each gene was measured using a calibration curve made from the standard samples as previously described.[11–14] Relative expression levels below 0.05 were regarded as missing values, possibly due to noise. Genes with missing values in more than 25% of cases were excluded from further analysis. Following conversion to a logarithmic scale, the data matrix was normalised by standardizing each sample to a median as 0. Missing values were estimated by the Bayesian principal component analysis.[17] Consequently, we selected 1453 genes for further analysis. Hierarchical cluster analysis was performed using GeneMath 2.0 software. Expression profiling data and supplemental information are available at our website (http://genome.mc.pref.osaka.jp/ATACPCR.html).

To predict peritoneal metastasis in an unknown test sample, we adopted a weighted voting (WV) algorithm, usually used in gene expression profiling.[18] WV algorithm is one of the classification methods between two classes; in this work

class 1 refers to the recurrence-free group and class 2 refers to peritoneal metastasis group. First, we calculated the signal-to-noise ratio (SNR), $S_i = (\mu_1 - \mu_2)/(\sigma_1 + \sigma_2)$, where μ and σ denote mean and standard deviation values of expression levels of a gene in each of the two classes and ranked genes based on the absolute value of SNR. Each gene was assigned a 'vote', which is the weighted difference between the gene expression level in a test sample and the average of the two classes: $v_i = S_i x(X_i - (\mu_1 + \mu_2)/2)$. The ultimate vote for a particular class assignment was computed by summing all weighted votes made by genes used in the class discrimination. The prediction strength (PS) was defined as $PS = (V_1 - |V_2|)/(V_1 + |V_2|)$, where V_1 is the summed votes exceeding the threshold (here 0), and V_2 is those less than the threshold, respectively. If $PS > 0$, we determined the test sample belongs to class 1. If $PS < 0$, we determined the test sample belongs to class 2. This model was evaluated by leave-one-out cross-validation, whereby one sample is withheld, a gene expression based model is trained by the remaining samples, and the model is then used to predict the class of the withheld sample.[18] The process was repeated to cover the entire sample, and the cumulative accuracy was then recorded. The leave-one-out cross-validation used in this study repeats the entire model-building process, including the selection of the important predictive genes, in each of the cross-validation sets.

Permutation testing, which involves randomly permuting class labels to determine gene-class correlations, was used to calculate statistical significance for selected genes.[18]

Statistical analysis was performed using Stat View 5.0J software. Relationships between recurrence and variable clinicopathological characteristics were evaluated by the chi square test. Peritoneal metastasis-free survival rate was calculated using the Kaplan–Meier method and compared by the log-rank test. Differences were considered to be significant at $P < 0.05$.

3. Results

3.1. Clinicopathological characteristics

Clinicopathological characteristics of both the learning and validation set are shown in Table 1. In the learning set, significant differences between the two groups were found in tumour size. In the validation set, no significant differences were found in any clinicopathological characteristics.

3.2. Construction of a prediction system for peritoneal metastasis

We performed hierarchical cluster analysis of the samples of the learning set using all 1453 genes. When the clinical samples were sorted on the basis of similarity of gene expression, the samples tended to be separated according to the information of peritoneal metastasis (Fig. 1).

Subsequently, we tried to construct a prediction system for peritoneal metastasis. First, we practiced WV algorithm based on SNR and evaluated the prediction accuracy by

Table 1 – Clinicopathological characteristics of patients

Characteristics		Learning set			Validation set		
		Recurrence(–) $n = 17$	Peritoneal metastasis(+) $n = 13$	P-value	Recurrence(–) $n = 11$	Peritoneal metastasis(+) $n = 13$	P-value
Gender	M	13	9		6	10	
	F	4	4	NS	5	3	NS
Age (median) location		60	65	NS	61	63	NS
	U	5	1		2	6	
	M	7	3		3	4	
	L	5	9	NS	6	3	NS
Tumour size	⩽5 cm	10	2		6	3	
	>5 cm	7	11	$P = 0.042$	5	10	NS
Procedure	Distal	8	7		6	4	
	Total	8	6		5	9	
	Proximal	1	0	NS	0	0	NS
Histology	Differentiated	8	2		4	4	
	Undifferentiated	9	11	NS	7	9	NS
Depth of invasion	ss	11	5		8	8	
	se	6	8		3	4	
	si	0	0	NS	0	1	NS
Lymphnode metastasis	N(–)	5	1		3	1	
	N(+)	12	12		8	12	NS
Adjuvant therapy				NS			
	Not performed	8	3		5	5	
	Performed	9	10	NS	6	8	NS
Follow-up time (median, months)		56	18		60	21	

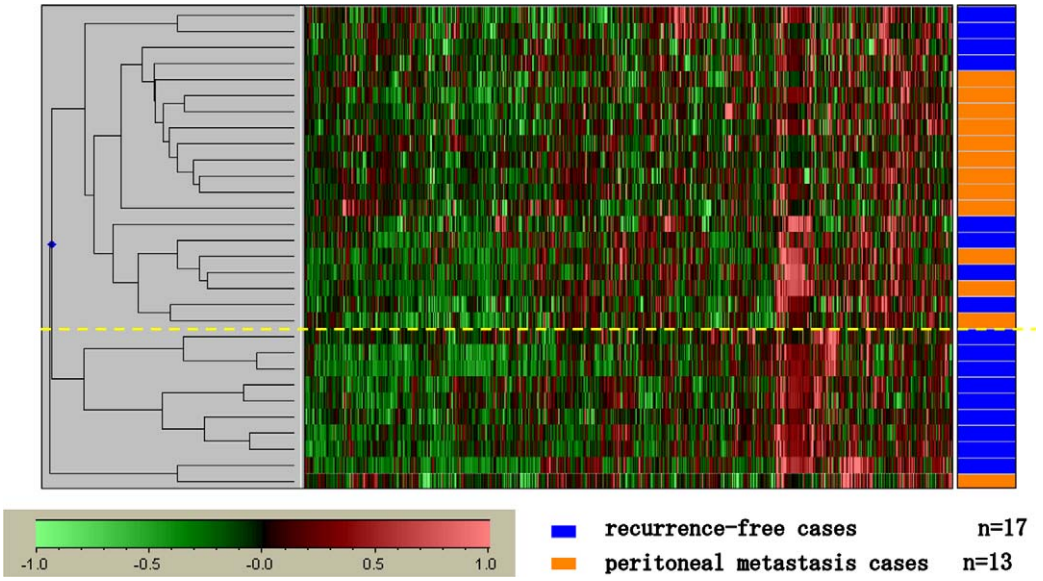

Fig. 1 – Hierarchical cluster analysis with 1453 genes in 30 cases of learning set. The rows and columns represent samples and genes, respectively. The color scale at the bottom indicates the relative expression levels in terms of standard deviations from the median.

leave-one-out cross-validation in the learning set. Leave-one-out cross-validation indicated the highest accuracy of 80% with the top 18 ranked genes. We adopted these most informative 18 genes as the predictive genes and constructed a prediction system for peritoneal metastasis by calculating SNR and the average of two classes of each 18 genes. The 18 diagnostic genes are summarised in Table 2. Nine genes were relatively upregulated in peritoneal metastasis cases, and nine genes were relatively downregulated in peritoneal metastasis cases, respectively. The *P* value of each gene was calculated by random permutation test with 50 000 random trials. The gene expression patterns of these 18 genes showed distinct profiles between two groups (Fig. 2).

3.3. *Evaluation of the prediction system in an additional data set*

To confirm the predictive performance, we validated our system with 24 additional patients of the validation set. Our system classified patients into either a 'good signature group',

Table 2 – List of 18 selected genes

Rank[a]	*P*-value[b]	Up/down[c]	GeneBank ID	Gene name
1	0.00018	Up	–	est
2	0.00068	Down	BC015799	caspase-7
3	0.00084	Down	NM_005125	Copper chaperone for superoxide dismutase (CCS)
4	0.00144	Up	–	est
5	0.00066	Down	BC009238	Tubulin alpha
6	0.00142	Up	NM_014453	BC-2 Putative breast adenocarcinoma marker (32 kDa)
7	0.0012	Down	NM_002490	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6.14 kDa (NDUFA6)
8	0.002	Down	BM990816	cDNA clone IMAGE:5863248 3′, mRNA sequence
9	0.00228	Down	NM_007158	NRASestrelated gene (D1S155E)
10	0.00106	Down	BC020516	Clone IMAGE:3882977, mRNA, partial cds.
11	0.0024	Up	NM_005147	DnaJ (Hsp40) homolog, subfamily A, member 3 (DNAJA3)
12	0.002	Up	NM_006083	IK cytokine, downestregulator of HLA II (IK)
13	0.0031	Up	NM_007209	Ribosomal protein L35 (RPL35)
14	0.00158	Up	NM_013230	CD24 antigen (small cell lung carcinoma cluster 4 antigen) (CD24)
15	0.00382	Up	NM_013442	Stomatin (EPB72)estlike 2 (STOML2)
16	0.00658	Up	NM_001087	Angioestassociated, migratory cell protein (AAMP)
17	0.00548	Down	NM_017816	Hypothetical protein FLJ20425 (LYAR)
18	0.0029	Down	AL832782	cDNA DKFZp686N107 (from clone DKFZp686N107)

a Ranking was according to signal-to-noise ratio.
b *P* values were calculated by random permutation test.
c Up or downregulation was defined as expression in peritoneal metastasis cases compared with recurrence-free cases.

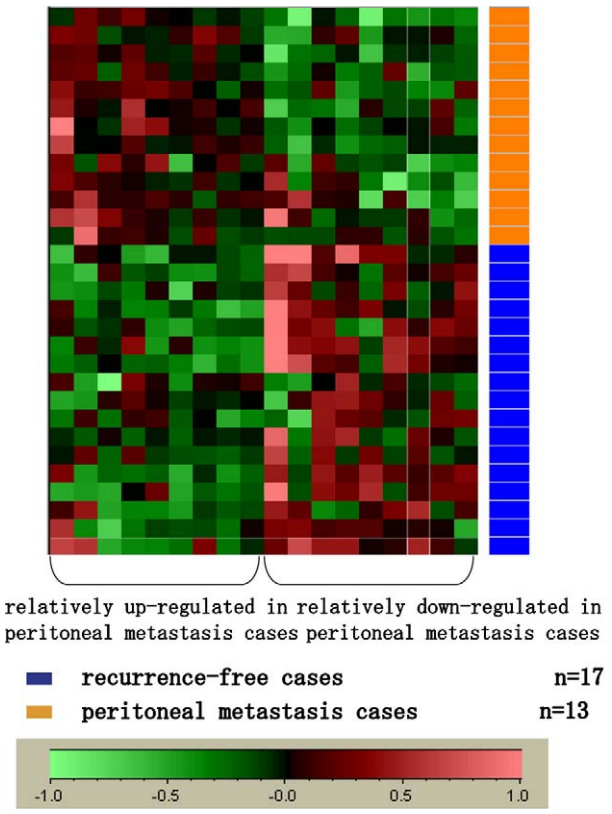

Fig. 2 – Gene expression pattern with informative 18 genes in 30 cases of learning set. The rows and columns represent samples and genes, respectively.

who were predicted to have no recurrence, or a 'poor signature group', who were predicted to have peritoneal metastasis. Our diagnostic system correctly predicted recurrence in 18 of 24 (75.0%) validation cases. The odds-ratio for the 'poor signature group' compared with the 'good signature group' was 8.89 (95% confidence interval (CI) 1.40–8.89, $P = 0.0433$). Kaplan–Meier analysis with peritoneal metastasis revealed a significant difference between the 'good signature group' and 'poor signature group' ($P = 0.0225$, Fig. 3).

4. Discussion

Peritoneal metastasis is the most frequent pattern of recurrence in patients with advanced gastric cancer. Cytologic examination of peritoneal lavages, though it has been applied to assess the risk of peritoneal metastasis, is reported to lack of sensitivity, because a large number of patients die of peritoneal metastasis even with negative cytologic results.[19] Therefore, new techniques, which identify high-risk cases for peritoneal metastasis, especially in patients after curative resection, are urgently required. Deaths caused by the recurrence of advanced gastric cancer after curative resection generally occur within three years of operation.[20] In the present study, we defined recurrence-free cases as those free of recurrence for at least three years after curative resection.

In the learning set, we constructed the prediction system for peritoneal metastasis by comparing gene expression profiling between patients with synchronous peritoneal metastasis and recurrence-free patients. Then, this system was applied to the independent validation set comprised of patients with metachronous peritoneal metastasis and recurrence-free patients and classified patients into either a 'good signature group' or a 'poor signature group' which revealed significant difference with regard to peritoneal metastasis by Kaplan–Meier analysis. The present results demonstrate that our prediction system is useful for identifying high risk patients for peritoneal metastasis after curative resection.

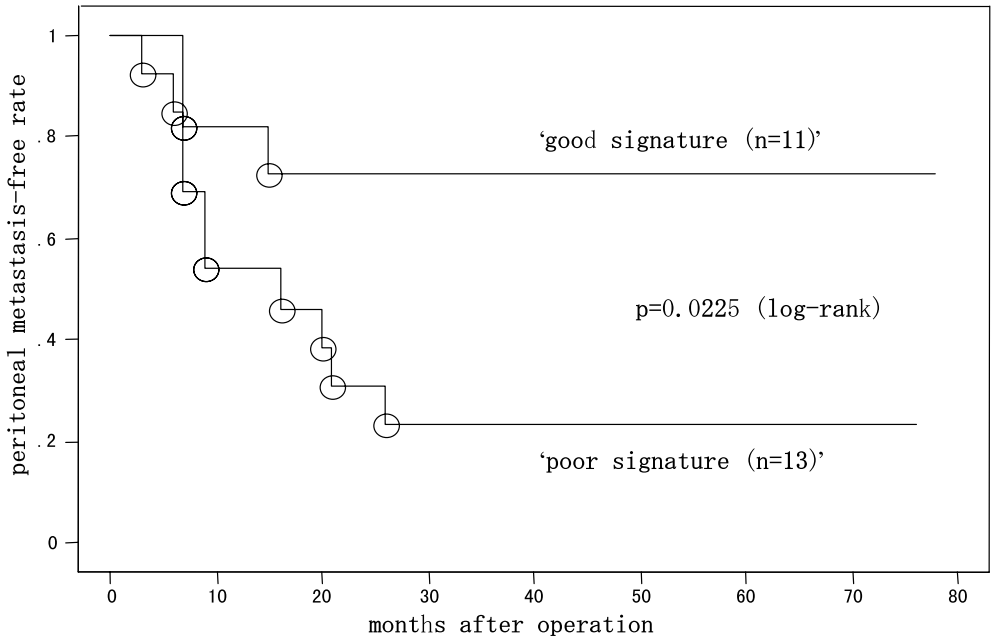

Fig. 3 – Peritoneal metastasis-free curves of patients in the validation set by the Kaplan–Meier method. Significant deference was observed between the 'good signature group' and 'poor signature group'.

Recently, several research groups applied RT-PCR for the detection of micrometastasis in the peritoneal cavity and reported that micrometastasis may influence recurrence or prognosis.[21] Coordination of multiple genes is involved in metastasis, whereas only one gene or a few genes have been the subject of these reports. Differences in the metastatic potential are expected to be due to a combination of differentially expressed genes. In this regard, Sakakura *et al.*[22] and Mori *et al.*[23] performed microarray analysis in gastric cancer cell lines and clinical samples of peritoneal lavages to select marker candidates for peritoneal metastasis. Both studies aimed to detect micrometastasis in peritoneal lavages. Peritoneal metastasis has been postulated that tumour cells in the gastric serosa develop the capability for implantation when they become exposed to the abdominal cavity. They then detach from the gastric wall and float within the abdominal cavity until they make contact with peritoneal mesothelium, stomata or milky spots, where they implant and proliferate within the host tissue. On the other hand, some investigators suggested that peritoneal metastasis may occur also through the lymphatic root.[24,25] In this study, to predict peritoneal metastasis we performed gene expression profiling of primary site as a means predicting peritoneal metastasis. Analysis of the primary site is considered reasonable because cancer cells in the abdominal cavity or lymphatic root are derived from the primary site. Our results suggested that the metastatic potential to the peritoneum is encoded in a primary site. Several gene expression profiling studies based on analysis of primary sites of cancer which accurately predicted the clinical outcome of individuals lend support to our result.[7,26]

Informative 18 genes selected for our prediction system included various genes reported to be associated with gastric cancer or other malignancies. *Caspase 7* encodes a protein which is a member of the cysteine-aspartic acid protease (caspase) family. *Caspase 7* is one of pathways responsible for apoptotic process in gastric cancer cell lines.[27] *HSP40*, which encodes a heat shock protein, has a molecular chaperone activity. *HSP40* has been implicated in gastric carcinogenesis[28] and is also selected as diagnostic gene in our previous study.[11] *CD24* is a small glycosylated mucin-like glycosylphosphatidyl-inositol-linked cell surface protein and an independent prognostic marker of shortened patient survival in various carcinomas.[29] Further biological studies on these genes are required for a full application of our findings.

High risk patients for peritoneal metastasis identified by our prediction system may be suitable candidates for post-operative adjuvant chemotherapy. In most cases, peritoneal metastasis after surgery is diagnosed at a measure of size. However, once peritoneal metastasis of visible size is established, complete cure is not possible at present. It is generally believed that the smaller the tumour size, the more effective the chemotherapy. To date, no definitive conclusions have been drawn from randomised clinical trials of adjuvant chemotherapy for gastric cancer.[30] However, the majority of previous clinical trials were designed for non-stratified patients and thus may have buried the potential benefit of adjuvant therapies. High risk patients for recurrence after sufficient local control may be the most eligible patients for clinical trials of post-operative adjuvant chemotherapy.

In summary, our prediction system using 18 genes identified by systematic analysis of gene expression profiling can predict peritoneal metastasis in patients with advanced gastric cancer after curative resection accurately. By combining our system with conventional clinicopathological factors, perhaps we could more accurately predict a patient's outcome. Because our prediction system involves only a small number of genes and a simplified algorithm that does not require statistical software or specialists, this system should easily lead to future clinical application.

Conflict of interest statement

None declared.

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