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Review Article

Application of MicroRNA in Diagnosis and Treatment of Ovarian Cancer

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Ovarian cancer has a poor prognosis because early detection is difficult and recurrent ovarian cancer is usually drug-resistant. The morbidity and mortality of ovarian cancer are high worldwide and new methods of diagnosis and therapy are needed. MicroRNAs (miRNAs) are posttranscriptional regulators of gene expression that are involved in carcinogenesis, metastasis, and invasion. Thus, miRNAs are likely to be useful as diagnostic and prognostic biomarkers and for cancer therapy. Many miRNAs have altered expression in ovarian cancer compared to normal ovarian tissues and these changes may be useful for diagnosis and treatment. For example, deficiencies of enzymes including Dicer and Drosha that are required for miRNA biogenesis may be adverse prognostic factors; miRNAs such as miR-214 and miR-31, which are involved in drug resistance, and the miR-200 family, which is implicated in metastasis, may serve as biomarkers; and transfection of downregulated miRNAs and inhibition of upregulated miRNAs may be effective for treatment of ovarian cancer. Chemotherapy targeting epigenetic mechanisms associated with miRNAs may also be effective to reverse gene silencing.

1. Introduction

Ovarian cancer is the eighth most common female cancer worldwide and ranks seventh in mortality. About 220,000 women are diagnosed with ovarian cancer each year and the disease causes about 140,000 deaths annually [1]. In Japan, the incidence and mortality of ovarian cancer have increased over the past 10 years [2, 3]. The 5-year survival rate for patients with advanced ovarian cancer is only 30%, despite the development of chemotherapy with platinum-based drugs and taxanes [4]. The high mortality is associated with difficulties in early detection because ovarian cancer rarely causes subjective symptoms and safe and minimally invasive procedures for early detection have not been established. Consequently, 40% to 50% of cases are detected in advanced stages III and IV. Another cause of the high mortality is resistance to chemotherapy. Ovarian cancer is highly responsive to initial anticancer treatment, but about half of the advanced cases recur within two years and have a decreased response to

chemotherapy, resulting in a poor prognosis [5]. For these reasons, there is an urgent need to develop new therapies, find clinically useful biomarkers, and identify new targets for treatment of ovarian cancer.

Many studies of ovarian cancer have focused on protein-coding genes. However, RNA molecules transcribed from noncoding genes also have biological functions. These noncoding RNAs include microRNAs (miRNAs) that cleave a target mRNA and repress translation of proteins, and some miRNAs show site- and stage-specific differences in expression in ovarian cancers. Many recent studies have shown that miRNAs are involved in suppression or progression of ovarian cancer. Therefore, miRNAs may be useful as diagnostic and prognostic biomarkers and also for therapy. Epigenetic therapy related to miRNAs may be particularly effective for resensitization of ovarian cancer cells to chemotherapy after development of resistance and recurrence. In this paper, we describe the possible use of miRNAs in diagnosis and treatment of ovarian cancer.

2. miRNAs Implicated in Ovarian Function

Ovarian function, particularly follicular development, is controlled by hormones such as gonadotropins, including follicle-stimulating hormone (FSH) and luteinizing hormone (LH). The ovary itself also produces sex hormones such as progesterone and estrogen, as well as cytokines of the transforming growth factor beta (TGF- β) superfamily [6]. A recent study suggested cross talk between the signals of local ovarian factors and endocrine system hormones including gonadotropins [7]. Thus, ovarian function is controlled by complex molecular signaling that maintains normal follicular development and atresia, in which protein expression is regulated quantitatively and temporally. Failure of the regulatory mechanisms is likely to lead to various diseases, including infertility.

Follicular cells are roughly classified into theca cells, granulosa cells, and oocytes. Granulosa cells proliferate in a FSH-dependent manner during follicular maturation and are involved in estrogen synthesis. Mase et al. found that many miRNAs in the let-7 family are expressed in human ovarian granulosa KGN cells, which maintain expression of FSH receptors. Genes targeted by the let-7 family include those involved in follicular maturation and atresia, suggesting involvement of miRNAs in these phenomena [8]. Murchison et al. produced Dicer 1 knockout mice with oocyte-specific deletion of Dicer, an important enzyme for miRNA biogenesis. In these mice, functional expression of miRNAs was completely deleted. Dicer deletion had no effect on early folliculogenesis but arrested the first meiotic division in oocytes associated with spindle and chromosomal aggregation hypoplasia. More than 2,000 mRNAs had significantly changed expression associated with these abnormalities. During oocyte maturation, including meiosis, gene transcription was completely repressed and only mRNAs inherited before maturation remained in cells. Expression of many mRNAs was affected in Dicer-deleted oocytes, suggesting direct or indirect posttranscriptional regulation by miRNAs [9].

3. Changes in miRNA Expression in Ovarian Cancer

Recent studies have identified many oncogenic miRNAs (oncomiRs) and tumor suppressor miRNAs (tumor suppressor miRs) (Table 1) [10–16]. Iorio et al. found several miRNAs with altered expression in ovarian cancer tissues compared with normal tissues, with miR-199a, miR-200a, miR-200b, and miR-200c having significantly increased expression and miR-140, miR-145, and miR125b1 showing markedly decreased expression in the cancer tissues. miR-140 is located at 6q22, a common defective chromosomal site in ovarian tumors, and this miRNA is thought to target genes associated with invasion, including matrix metalloproteinase 13, fibroblast growth factor 2, and angiogenic VEGFA [10]. Bracken et al. showed that miR-429, miR-200a, and miR-200b are regulated by ZEB1 and SIP1, which are inhibitors of the epithelial-mesenchymal transition (EMT), and that miR-200a and miR-200b negatively regulate expression of ZEB1 and SIP1, providing a negative feedback loop [11].

TABLE 1: Changes in miRNA expression in ovarian cancer.

Upregulated (oncomiRs)	Downregulated (tumor suppressor miRs)
miR-199a	miR-140
miR-200a	miR-145
miR-200b	Let-7i
miR-200c	miR-15
miR-429	miR-16
	miR-373
	miR-520c
	miR-125b1

With regard to miRNA processing of mRNA, an interesting study of the relationship of ovarian cancer with miRNAs by Merritt et al. [12] showed that the mRNA levels of Dicer and Ribonuclease 3 (Drosha) decreased in 60% and 51% of tissue samples from 111 patients with invasive epithelial ovarian cancer. Downregulation of Dicer was significantly related to tumor stage progression and downregulation of Drosha was significantly related to a suboptimal residual tumor size >1cm after cytoreductive surgery. Conversely, patients with high levels of Dicer and Drosha in cancer tissues had significantly prolonged median survival times. Cells with downregulation of Dicer and Drosha are likely to have lower levels of mature miRNAs, which suggests that certain miRNAs are involved in progression of ovarian cancer [12].

4. miRNAs Associated with Drug Resistance

A total of 27 miRNAs have been associated with responsiveness to chemotherapy [13]. Yang et al. found that miR-214, which targets PTEN, is frequently expressed in ovarian cancer tissues and that let-7i, which enhances resensitization to platinum resistance, is expressed less in the same tissues [14]. Mitamura et al. showed that control of MET expression by miR-31 is involved in drug-resistance mechanism in paclitaxel-resistant ovarian cancer cells [15]. Aqeilan et al. found that miR-15 and miR-16 cause cellular resistance to many drugs through targeting the BCL2 gene [16]. Leskelä et al. showed that the miR-200 family (miR-141, miR-200a, miR-200b, miR-200c, and miR-42) is implicated in the response to paclitaxel treatment and progression-free survival via β tubulin III regulation. In particular, miR-200c is significantly associated with recurrence of ovarian cancer and miR-429 is associated with progression-free and overall survival rates [17].

Key drugs against ovarian cancer are taxanes and cisplatin. Boyerinas et al. found that let-7g and let-7a are involved in drug resistance [18]. Let-7g suppresses IMP-1, which is involved in multidrug resistance and increased sensitivity to taxanes. The expression level of let-7a is a potential marker for choosing chemotherapeutic agents, since patients with extremely low let-7a expression are responsive to platinum-based drugs and paclitaxel, whereas those with high levels of let-7a had increased survival only in monotherapy with a platinum-based drug [19]. Nagaraja et al. and

TABLE 2: Histological types and miRNA expression in ovarian cancer.

Tissue type	Upregulation			Downregulation		
Serous adenocarcinoma	miR-7	miR-200a/c		miR-148b		
	miR-22	miR-302b		miR-211		
	miR-373	miR-34c-5p		miR-31		
	miR-449a	miR-146b-5p				
Endometrioid adenocarcinoma	miR-9	miR-183	miR-205	miR-22	miR-222	miR-324-3p
	miR-96	miR-196a	miR-212	miR-101	miR-299-5p	miR-325
	miR-182	miR-196b	miR-375	miR-194	miR-302b	miR-373
	miR-141	miR-200a/b/c				
Clear cell adenocarcinoma	miR-29b	miR-200a/c		miR-20a		
	miR-30a	miR-486-5p				
	miR-30e					
Mucinous adenocarcinoma	miR-141	miR-200b				
Undifferentiated carcinoma				miR-9		
				miR-18		

Peng et al. showed that miR-100, a tumor suppressor miRNA, increased sensitivity to everolimus, an anticancer drug [20, 21]. miR100 is also an independent predictor of overall survival in patients with ovarian cancer. Hong et al. showed that miR-376c suppresses signaling of Nodal/activin receptor-like kinase 7 (ALK7), which is involved in drug sensitivity, and decreases the effects of cisplatin and carboplatin [13]. Fu et al. found that miR-93 targets integrin and enhances tumor growth, angiogenesis, and the resistance for cisplatin [22].

5. Utility of miRNAs in Diagnosis of Ovarian Cancer

Many miRNAs have altered expression levels in ovarian cancer compared to normal tissues. In addition, changes in miRNA levels are dependent on and related to the ovarian cancer tissue type, stage, histological type, prognosis, and drug resistance (Table 2) [8, 10, 23–30]. These findings suggest the possibility of early diagnosis of ovarian cancer using miRNAs. In the miR-200 family, Boyerinas et al. showed that miR-200a and miR-200c are expressed in serous adenocarcinoma, clear cell adenocarcinoma, and endometrioid adenocarcinoma, and miR-200b and miR-141 occur in endometrioid adenocarcinoma and mucinous adenocarcinoma [18]. Toloubeydokhti et al. found decreased expression of miR-212 in serous cystadenoma [31]. Target genes of miR-212 include those with overexpression in this histological type of ovarian cancer and mutated genes in hereditary ovarian cancer. Therefore, miR-212 may be a marker for differentiating ovarian cancer. Downregulation of miR-31, a tumor suppressor miRNA, has been shown in serous adenocarcinoma, and miR-31 suppresses expression of cell cycle regulatory factors via p53 [32]. Expression of miR-373 is variable in undifferentiated carcinoma [33], but the target genes and function of this miRNA are unknown. Overexpression of miR-21 in clear cell carcinoma has been shown to cause downregulation of PTEN [34].

6. Utility of miRNAs in Treatment of Ovarian Cancer

Treatment options for ovarian cancer include supplementation of miRNAs that are downregulated in cancer tissue for recovery of function and inhibition of the function of upregulated miRNAs by administration of complementary nucleic acids. Garzon et al. showed that the effects of upregulated oncomiRs could be suppressed using an antagomir, an oligonucleotide complementary to the miRNA administered as an antisense oligonucleotide or LNA [35]. Lu et al. developed an anti-miRNA antisense oligodeoxyribonucleotide (MTG-AMO) for suppression of many miRNAs, including miR-21, and showed that this was effective in cancer with concurrent multiple mRNA abnormalities [36]. Dai et al. established a therapy for ovarian cancer based on targeted delivery of miR-29a to cancer tissues for the purpose of reexpressing PTEN, a tumor suppressor. The potential antitumor effect of a miR-29a-transfected chimera was apparently based on expression of downstream molecules and apoptosis of ovarian cancer cells [37].

The association of miRNAs with peritoneal metastasis, the major cause of death in patients with ovarian cancer, has also been studied. Ohyagi-Hara et al. found that integrin $\alpha 5$, a fibronectin receptor, increased the adhesion of cancer cells and induced metastasis and focused on the inverse correlation of integrin $\alpha 5$ and miR-92a levels. Transfection of ovarian cancer cells with miR-92a reduced expression of integrin $\alpha 5$ and suppressed peritoneal metastasis [38]. Cittelly et al. found that recovery of the level of miR-200c, which is known to increase sensitivity to platinum-based anticancer drugs, by transfection suppressed carcinogenesis and decreased the number of cancer cells. Recovery of miR-200c in combination with paclitaxel also decreased the cancer cells in established tumors. These results suggest that recovery of miR-200c immediately before highly cytotoxic chemotherapy improves the treatment response or reduces the effective dose of the anticancer drugs [39]. These outcomes show

that miRNA transfection has an antitumor effect. Transfected miRNAs are synthetic nucleic acids that require specific modes of administration [40–42]. These approaches include intravenous administration of a complex with atelocollagen, nanoparticles with cell-specific targeting, and conjugation with RVG peptide for crossing the blood-brain barrier. Gene therapy for introduction of miRNAs may also be useful if safety can be confirmed.

Epigenetic therapy has attracted attention as an alternative to classical approaches such as miRNA transfection. Acquisition of drug resistance reduces the survival rate in cancer and many cases of ovarian cancer are resistant to platinum-based anticancer drugs. This resistance is associated with miRNAs and various drug-resistance genes induced by methylation and signaling gene silencing. However, epigenetic changes are reversible, in contrast to gene mutations, and there is a potential to reverse gene silencing using DNA methyltransferase (DNMT) inhibitors, which are drugs that prevent hypermethylation by irreversibly binding to the active site of DNMT [43]. These drugs are effective as monotherapy for hematologic malignancy [44, 45], but not in solid cancer. However, effects on solid cancer are likely to be found in combination with other drugs. In ovarian cancer cells, DNMT inhibitors induce hypomethylation and reverse resistance to platinum-based anticancer drugs. Phases I and II clinical trials of decitabine, a DNMT inhibitor, are ongoing in ovarian cancer [46, 47]. Matei et al. found that decitabine in combination with carboplatin restored the expression of silenced tumor suppressor genes and may contribute to resensitization of platinum-resistant endometrial cancer. A phase I clinical trial of decitabine has shown that combined administration with carboplatin is safe and decreases methylation of multiple genes *in vivo* [48].

Malignant tumors, including ovarian cancer, include cancer initiation cells and cancer stem cells, which are referred to as cancer progenitor cells and are involved in development of drug resistance [49–51]. Chemotherapy targeting mitosis cannot eliminate all cancer stem cells during cell cycle arrest or low activity conditions, and residual cells promote regrowth of the tumor. Epigenetic therapy stabilizes differentiation and may target undifferentiated cancer stem cells. Thus, targeting of epigenetic mechanisms is likely to improve outcomes in ovarian cancer.

7. Conclusion

miRNAs have attracted significant interest, but the history of this field is relatively short and many issues remain to be resolved. Clinical studies of miRNAs have just started, but functional genomic analyses have produced results that may lead to clinical applications in the near future. Early diagnosis of ovarian cancer is important to improve treatment outcomes, and profiling using miRNA arrays may contribute to the detection of tissue type, stage, and prognosis. Induction of apoptosis of cancer cells using miRNAs may be a basic treatment strategy for reduction of metastasis, including peritoneal metastasis, and decreasing resistance to platinum-based anticancer drugs. Recovery of tumor suppression effects may be possible by transfection of miRNAs

downregulated in cancer tissues or by suppression of upregulated miRNAs. miRNA expression may also be modified by targeting epigenetic mechanisms such as through reversal of hypermethylation. These potential treatment approaches will require further basic studies to facilitate drug discovery.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Simultaneous Analysis of the Gene Expression Profiles of Cancer and Stromal Cells in Endometrial Cancer

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To address the role of cancer-stroma interactions, we performed gene expression profiling of both cancer and stroma, using matching samples of endometrial cancer (EC), and analyzed the relationship between the gene expression pattern and prognosis in EC. Sixty EC cases were included in this study (38 nonrecurrent and 22 recurrent). Cancer and stroma were separated by performing laser capture microdissection, and microarray analysis was performed separately on cancer and stromal cells. Genes related with progression-free survival (PFS) in cancer and stroma were analyzed using the Cox regression model, and we established a formula, based on the gene expression pattern of cancer and stroma, to predict recurrence using logistic regression. We estimated the accuracy of the formula using the 0.632 method. All cases were classified based on the 79 selected genes of cancer and stroma related to PFS, based on unsupervised clustering. A total of 143 genes in cancer, and 79 genes in stroma were significantly related with PFS. The estimated area under the curve of receiver operating characteristics curve in cancer and stroma to predict recurrence were 0.800 and 0.758, respectively. Based on the 79 genes of cancer, the 22 recurrent cases were divided into two groups, which generally correlated with the histological grade. In contrast, based on the 79 genes of stroma, the 22 recurrent cases displayed homogeneous gene expression, unrelated to the histological grade. We conclude that gene expression profiles of cancer and stroma can predict the recurrence of EC and stromal that gene expression does not depend on the cancer grade. © 2014 Wiley Periodicals, Inc.

INTRODUCTION

Endometrial cancer (EC) is the most common cancer of the female genital tract; however, the molecular mechanisms behind its progression are not fully understood. Tumors are heterogeneous and consist of founder cancer cells together with stromal cells, including blood and lymph endothelial cells, inflammatory cells, immunocytes, macrophages, and fibroblasts. Stroma is thought to play a role in tumor behavior, including invasion and metastasis, and response to therapy, and cancer-stroma interactions are important for cancer progression (Hanahan and Weinberg, 2000; Bissell and Radisky, 2001; Mueller and Fusenig, 2004; Bhowmick et al., 2004; Kalluri and Zeisberg, 2006). However, the role of stroma in the development and progression of epithelial neoplasia has not been thoroughly investigated. Finak et al. (2008) used laser capture microdissection (LCM) to

compare gene expression profiles of tumor stroma from 53 primary breast cancers, and established a new stroma-derived prognostic predictor (SDPP) that stratifies disease outcomes independently of standard clinical prognostic factors and published gene expression-based predictors. We previously performed gene expression profiling using microdissected tissues to identify genes expressed in

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stroma, which could provide prognostic information in epithelial ovarian cancer, and reported that early growth response 1 (EGR1) expression, not only in cancer but also in stroma, was related to the prognosis of ovarian cancer. In agreement with this, the EGR1 protein was found in the nuclei of cancer cells, as well as in fibroblasts (Kataoka et al., 2012). Based on these findings, we speculated that some genes are associated with tumor-stroma interactions, and accordingly, a number of studies have recently demonstrated that stromal gene expression patterns are related with prognosis in breast cancer, ovarian cancer, non-small cell lung cancer, and prostate cancer (Finak et al., 2008; Farmer et al., 2009; Gregg et al., 2010; Navab et al., 2011; Kataoka et al., 2012). To address the role of cancer-stroma interactions, it is important to analyze the gene expression pattern of both cancer and stroma, using the same sample. However, to the best of our knowledge, no previous studies have examined the gene expression profile of cancer and stroma using this method. Hence, we here microdissected cancer and stroma from EC using LCM, performed gene expression profiling, and analyzed the relationship between the expression profile pattern and prognosis, simultaneously in cancer and stroma.

MATERIALS AND METHODS

Patients and Samples

Subjects eligible for this study were patients with histologically confirmed EC. Additional inclusion criteria included an Eastern Cooperative Oncology Group performance status of 0 to 2. Exclusion criteria included a history of prior chemotherapy or major surgery. All surgical staging and debulking procedures were performed by experienced gynecologists. Histologic diagnosis was confirmed through microscopic examination of the hematoxylin and eosin (H&E)-stained sections, according to World Health Organization criteria. Clinical stages were determined according to the International Federation of Gynecology and Obstetrics system. Progression-free survival (PFS) was followed and analyzed after the patients had received primary surgery. This study was approved by the Institutional Review Board of the Osaka City General Hospital, National Defence Medical College, and School of Medicine, Keio University, and written informed consent was

obtained from all patients. Specimens obtained at operation were immediately stored at -80°C .

Microdissection, RNA Extraction, and Amplification of RNA

Microdissection was performed as described previously (Kataoka et al., 2012). In brief, frozen sections ($6\ \mu\text{m}$) prepared from tumor tissue specimens were affixed to glass slides and stained by HistogeneTM LCM Frozen Section Staining Kit (Arcturus Engineering, Mountain View, CA). Stained sections were microdissected using a Pix-Cell Iie LCM system (Arcturus Engineering). Tumor cells and adjacent nontumor stromal cells were visualized under the microscope and selectively detached by activation of the laser. Cancer and stromal tissues within 200 cells from the margin of tumors were dissected for each case. Total RNA extraction was performed with the PicoPureTM RNA Isolation Kit according to the manufacturer's instructions (Arcturus Engineering). RNA was amplified using a modified single round T7 RNA amplification protocol. In brief, total RNA ($600\ \text{ng}$) was first incubated with $1\ \mu\text{l}$ of T7 primer (5'-GCATTAGCGGCCGCGAAATTAATACGACTCACTATAGGGAGATTTT'TTT'TTT'TTT'TTT'TTT'TTVN-3', $200\ \text{ng}/\mu\text{l}$) in a total volume of $50\ \mu\text{l}$ for 3 min at 70°C . First strand cDNA synthesis was subsequently performed by incubating $5\ \mu\text{l}$ of primer-annealed sample and $5\ \mu\text{l}$ of first strand master mix containing $2\ \mu\text{l}$ of $5\times$ first strand buffer, $1\ \mu\text{l}$ of $0.1\ \text{M}$ dithiothreitol (DTT), $0.5\ \mu\text{l}$ of diethylpyrocarbonate (DEPC)-treated water, $0.5\ \mu\text{l}$ $10\ \text{mM}$ deoxyribonucleotide triphosphate (dNTP) mix, $0.5\ \mu\text{l}$ RNase Inhibitor, and $0.5\ \mu\text{l}$ of M-MLV reverse transcriptase ($200\ \text{U}/\mu\text{l}$) for 75 min at 37°C . Next, second strand cDNA synthesis was performed by incubating the $10\ \mu\text{l}$ first strand reaction with $65\ \mu\text{l}$ of second master mix, which contained $46\ \mu\text{l}$ DEPC-treated water, $15\ \mu\text{l}$ $5\times$ second strand buffer, $1.5\ \mu\text{l}$ of $10\ \text{mM}$ dNTP mix, $0.5\ \mu\text{l}$ of *Escherichia coli* DNA Ligase ($10\ \text{U}/\mu\text{l}$), $1.5\ \mu\text{l}$ *E. coli* DNA Polymerase I ($10\ \text{U}/\mu\text{l}$), and $0.5\ \mu\text{l}$ *E. coli* RNase H ($2\ \text{U}/\mu\text{l}$), for 2 hr at 16°C , followed by 15 min at 70°C . The entire $75\ \mu\text{l}$ cDNA sample was loaded onto a ChromaSpin TE-200 spin column (BD Biosciences, San Diego, CA), and centrifuged for 5 min at $2,900\ \text{rpm}$ in an Eppendorf centrifuge. Purified cDNA was collected, lyophilized, dissolved in $8\ \mu\text{l}$ of RNase-free water, and incubated at 70°C for 10 min. In vitro transcription was subsequently performed by incubating the

8 μ l post-lyophilization cDNA product with 12.2 μ l of master mix containing 2 μ l of 10 \times T7 Reaction Buffer, 6 μ l of 25 mM rNTP Mix, 2 ml of 100 mM DTT, 0.2 μ l of RNase Inhibitor (40 U/ml), and 2 μ l of T7 RNA Polymerase for 3 hr at 37°C. The amplified RNA was purified on an RNeasy mini column (Qiagen, Valencia, CA) according to the manufacturer's protocol. The purified amplified RNA was quantified with RiboGreen RNA Quantitation Reagent (Molecular Probes, Eugene, OR).

Oligonucleotide Microarray Study

The microarray procedure was performed according to the protocol, described by the manufacturer (Illumina Inc., San Diego, CA). In brief, total RNA was checked for quality using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and cDNA was synthesized using the Illumina WG-DASL assay kit for IVT and DASL-based labeling. Hybridization was performed using the Human-Ref8 v3.0 Beadchips (Illumina Inc.) for 16 hr at 58°C. Signal intensities were measured with an Illumina BeadArray™ Reader and converted to numerical data using the Genome Studio Expression Module v1.6.0 (Illumina Inc.).

Statistical Analysis of Microarray Expression Data

All the microarray data were normalized using asinh (hyperbolic arc sine) transformation, which is a modified version of Huber's normalization with variance stabilization (Huber et al., 2002; Kurahashi et al., 2007). First, any differentially expressed genes related to PFS were identified in cancer and stroma, using the Cox regression model. Genes with a *P*-value less than 0.05 were considered statistically significant. Next, we established a formula for predicting the recurrence of EC based on the gene expression in the associated cancer and stromal cells: we used logistic regression to score and rank each identified gene based on its statistical significance in predicting recurrence, in a model that included the gene expression level. The established formula was cross-validated using the Efron's 0.632 bootstrap method (Efron, 1983). Receiver operating characteristic (ROC) curves were plotted according to the error rate adjusted using the 0.632 bootstrap method.

We classified all cases based on the gene expression of cancer and stroma using unsupervised classification, and developed a method for classifying the 60 EC cases based on the gene expression

profile of the top 79 selected genes in cancer and stroma. Finally, we performed ontology analysis.

RESULTS

Clinicopathological Features of EC Patients

The 60 specimens analyzed in this study were obtained from patients who were newly diagnosed with EC from 2000 to 2006 at Osaka City General Hospital, National Defence Medical College, and School of Medicine, Keio University, Japan. The median age of the patients was 55 years (range, 25–81). Thirty-nine patients were classified as having stage I EC (Ia, 12; Ib, 27), three as stage II, 13 as stage III (IIIa, 3; IIIb, 2; IIIc1, 5; IIIc2, 3), and five as stage IVb. Three tumors were adenoachantoma and 57 were endometrioid type EC. Nineteen samples were classified as histological grade 1 (G1), 31 as G2, and 10 as G3. Thirty-eight cases were nonrecurrent, and 22 were recurrent.

Thirty-six patients received abdominal simple hysterectomy + bilateral salpingo-oophorectomy + peritoneal washing, pelvic lymphadenectomy, and paraaortic lymphadenectomy; 11 patients received abdominal radical hysterectomy + bilateral salpingo-oophorectomy + peritoneal washing, pelvic lymphadenectomy, and paraaortic lymphadenectomy; six patients received abdominal semiradical hysterectomy + bilateral salpingo-oophorectomy + peritoneal washing, pelvic lymphadenectomy, and paraaortic lymphadenectomy; four patients received abdominal simple hysterectomy + bilateral salpingo-oophorectomy + peritoneal washing and pelvic lymphadenectomy; two patients received abdominal extended hysterectomy + bilateral salpingo-oophorectomy + peritoneal washing, pelvic lymphadenectomy, and paraaortic lymphadenectomy; and one patient received abdominal extended hysterectomy + bilateral salpingo-oophorectomy.

In 54 of 60 patients, complete resections were achieved. Out of the 60 patients, 50 patients received adjuvant chemotherapy (cisplatin + doxorubicin + cyclophosphamide, 37; carboplatin + paclitaxel, 13) after the initial surgery. The median follow-up period of all cases was 1,830 days (range, 120–3,375 days), and 38 patients were alive without relapse at follow-up (median, 2,023 days; range, 1,830–3,375 days).

Identification of Candidate Disease Progression-Related Genes by Performing Microarray Analysis

To identify candidate PFS-related genes, microarray analysis was performed on 60 samples.

TABLE 1. Seventeen Genes Included in Genes Lists of Both Cancer and Stroma Gene Ontology Terms for Genes

No.	Gene	Location	Biological process
1	ACRVI	11q24.2	Cell communication/signal transduction
2	ARHGAP4	Xq28	Cell communication/signal transduction
3	CLDN6	16p13.3	Cell communication/signal transduction
4	GPC5	13q32	Cell communication/signal transduction
5	HUNK	21q22.1	Cell communication/signal transduction
6	MPP6	7p15	Cell communication/signal transduction
7	PRCC	1q21.1	Cell communication/signal transduction
8	PTPRT	20q12-q13	Cell communication/signal transduction
9	RASAL2	1q24	Cell communication/signal transduction
10	S100A1	1q21.1	Cell communication/signal transduction
11	C16orf73	16p13.3	Biological process unknown
12	PCDHGA1	5q31	Biological process unknown
13	KIAA0514	10q11.22	Biological process unknown
14	FBXW4	10q24	Protein metabolism
15	NAT12	14q22.3	Metabolism:Energy Pathway
16	SPC25	2q31.1	Apoptosis
17	SUSD4	1q41	Immuno response

A total of 18,401 genes passed the filtering criteria and were further analyzed. A total of 2,336 genes in the cancer cells and 1,593 genes in the stromal cells were significantly related with PFS, with a *P*-value of <0.01 (Supplementary Information Table 1). Out of the 2,336 genes identified in cancer, 1,901 genes were upregulated and 435 genes were downregulated in recurrent cases, whereas in the stroma, 1,305 genes were upregulated and 288 genes were downregulated in recurrent cases.

Furthermore, 143 genes in cancer and 79 genes in stroma significantly correlated with PFS, with a *P*-value of <0.001. Of all genes, 17 genes (*ACRVI*, *ARHGAP4*, *C16orf73*, *CLDN6*, *FBXW4*, *GPC5*, *HUNK*, *KIAA0514*, *MPP6*, *NAT12*, *PCDHGA1*, *PRCC*, *PTPRT*, *RASAL2*, *S100A1*, *SPC25*, and *SUSD4*) were included in gene lists of both cancer and stroma. Of these 17 genes, 10 genes (*ACRVI*, *ARHGAP4*, *CLDN6*, *GPC5*, *HUNK*, *GPC5*, *MPP6*, *PRCC*, *PTPRT*, *RASAL2*, and *S100A1*) were related to cell communication/signal transduction (Table 1).

We analyzed the characteristics of the top 79 genes in cancer and stromal cells, according to the following Gene-Ontology categories (Ashburner et al., 2000; biological process, cellular role, and molecular function), and these are summarized in Tables 2 and 3 and Supplementary Information Table 2. Apoptosis-related genes tended to be common in cancer, whereas immunoresponse-related and regulation of nucleobase, nucleoside, nucleotide, and nucleic acid metabolism-related genes were commonly observed in stroma.

Prognostic Accuracy of the Established Formula Based on the Gene Expression Profile of Cancer Cells and Cancer-Stromal cells

We established a formula based on the gene expression profile of cancer and stroma to predict the recurrence using a logistic regression model. We estimated the accuracy of the formula using the 0.632 method. The ROCs of cancer and stroma to predict the recurrence are estimated in Figure 1. The estimated areas under the curve (AUC) of ROC in cancer and stroma, which were used to predict recurrence, were 0.800 and 0.758, respectively. The moderate accuracies to predict recurrence were achieved by the formula based on the gene expression profile of cancer and stroma.

Unsupervised Clustering of 60 EC Cases, Based on the 79 Genes Related to PFS

We classified all EC cases based on 79 selected genes related to PFS of cancer and stroma, by unsupervised clustering. Based on the gene expression patterns of cancer, the 38 nonrecurrent cases, excluding two cases, showed almost homogeneous expression, whereas the 22 recurrent cases were divided into two groups, excluding three cases (Fig. 2A): one group included 12 cases (G1,1; G2, 9; G3, 2) and the other group included seven cases (G2, 4; and G3, 3). Based on the 79 genes expression pattern of stroma, both the 38 nonrecurrent cases and the 22 recurrent cases displayed homogeneous gene expression, excluding three cases (Fig. 2B).

TABLE 2. The Top 79 Genes Related with Recurrence (Cancer)

No.	Gene	Location	P-value	Hazard ratio	CI of HR	Biological process
1	TCHHL1	1q21.3	0.000002	1.31E + 05	972–1.77E + 07	Cell communication/ signal transduction
2	ABCA4	1p22	0.000002	4.8	2.5–9.22	Metabolism/Energy pathways
3	SPC25	2q31.1	0.000004	12.9	4.34–38.48	Apoptosis
4	SAMD12	8q24.12	0.000006	8.29E + 73	6.30E+41–1.09E + 106	Cell communication/ signal transduction
5	ZFP42	4q35.2	0.000007	1.40E + 47	4.00E+26–4.90E + 67	Biological process unknown
6	C7orf52	7q22.1	0.000007	9.76	3.6–26.4	Biological process unknown
7	NT5M	17p11.2	0.000008	54.6	9.48–315	Metabolism/Energy pathways
8	SLC35D3	6q23.3	0.000008	1980	70.3–5.55E + 04	Biological process unknown
9	FRMPD1	9p13.2	0.000009	7.87	3.17–19.5	Biological process unknown
10	CDCA1	1q23.3	0.000009	21100	263–1.70E + 06	Cell communication/ signal transduction
11	TTC22	1p32.3	0.000013	11.7	3.86–35.3	Biological process unknown
12	IFRG15	1q25.2	0.000029	143	13.9–1.46E + 05	Biological process unknown
13	KIAA0514	10q11.22	0.000032	5.58	2.48–12.6	Biological process unknown
14	GIMAP5	7q36.1	0.000044	2.61E-71	3.57E-105–1.90E-37	Apoptosis
15	ALS2CR11	2q33.1	0.000044	8.12	2.98–22.2	Biological process unknown
16	SCUBE3	6p21.3	0.000048	16.2	4.23–62.3	Biological process unknown
17	KRTAP4-1	17q12-q21	0.000051	3.04E + 07	7320–1.27E + 11	Cell growth and/or maintenance
18	NR2E1	6q21	0.000051	2.38E + 05	596–9.54E + 07	Cell communication/ signal transduction
19	CLDN6	16p13.3	0.000053	3.7	1.95–6.89	Cell growth and/or maintenance
20	DLEU2L	1p31-p22	0.000053	57700	282–1.18E + 07	
21	SYTL2	11q14	0.000054	9.15	3.12–26.8	Regulation of exocytosis
22	C16orf75	16p13.13	0.000056	9.84	3.23–29.9	Biological process unknown
23	USH1G	17q25.1	0.000056	230	16.3–3.24E + 03	Biological process unknown
24	RDHE2	8q12.1	0.000063	1.08E + 05	370–3.13E + 07	Metabolism;Energy pathways
25	PPP1R11	6p21.3	0.000063	3.00E-03	1.74E-04–5.16E-02	Biological process unknown
26	ZNF382	19q13.12	0.000083	4.23E + 06	2130–8.42E + 09	Apoptosis
27	SP6	17q21.32	0.000085	8.77	2.97–25.9	Regulation of nucleo- base, nucleoside, nucleotide and nucleic acid metabolism
28	BMP7	20q13	0.000085	319	18–5.65E + 03	Cell communication/ signal transduction
29	HSF2BP	21q22.3	0.000091	12.1	3.47–42	Regulation of nucleo- base, nucleoside, nucleotide and nucleic acid metabolism

TABLE 2. (Continued)

No.	Gene	Location	P-value	Hazard ratio	CI of HR	Biological process
30	POLE2	14q21-q22	0.000098	206	14.1–3.01E + 03	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
31	RASAL2	1q24	0.000101	354	18.4–6.82E + 03	Cell communication/signal transduction
32	SPRR3	1q21-q22	0.000110	53	7.09–396	Cell growth and/or maintenance
33	MAGEA10	Xq28	0.000111	27.6	5.13–149	Immune response
34	C8orf15		0.000111	1.30E + 06	1030–1.63E + 09	
35	OVOL1		0.000125	108	9.86–1.18E + 03	
36	GAL3ST3	11q13.1	0.000130	5.42	2.28–12.9	Metabolism:Energy pathway
37	MPP6	7p15	0.000135	4.91	2.17–11.1	Cell communication/signal transduction
38	GDPD2	Xp13.1	0.000138	1.93E + 09	3.24E + 04–1.15E + 14	Cell communication/signal transduction
39	PTPRT	20q12-q13	0.000139	15.7	3.81–64.6	Cell communication/signal transduction
40	KRT39	17q21.2	0.000143	1.22E + 46	2.16E + 22–6.93E + 69	Cell growth and/or maintenance
41	CST5	20p11.21	0.000153	182	12.3–2.69E + 03	Protein metabolism
42	PJCG6		0.000155	8.8	2.85–27.2	
43	CCBL2	1p22.2	0.000173	546	20.4–1.46E + 04	Metabolism:Energy pathway
44	OR4F3	5q35.3	0.000187	60.1	7.01–516	Cell communication/signal transduction
45	ADAM23	2q33	0.000193	2.65	1.59–4.42	Protein metabolism
46	FLJ41046		0.000198	2350	39.4–1.40E + 05	
47	ZNF829	19q13.12	0.000202	3.63E + 10	9.77E + 06–1.34E + 16	
48	CPN2	3q29	0.000210	2820	42.3–1.89E + 05	Protein metabolism
49	NOS1	12q24.2-q24.31	0.000212	18.2	3.92–84.3	Metabolism:Energy pathway
50	OR11H1	22q11.2	0.000215	5.96E + 13	3.02E + 06–1.18E + 21	Cell communication/signal transduction
51	KCNMB3	3q26.3-q27	0.000227	99.7	8.63–1.15E + 03	Toransport
52	N4BP3	5q35.3	0.000230	1780	33.2–9.54E + 04	
53	TSPAN16	19p13.2	0.000241	200	11.8–3.38E + 03	Cell communication/signal transduction
54	ACRV1	11q24.2	0.000256	18.1	3.84–85.8	Cell communication/signal transduction
55	HUNK	21q22.1	0.000258	298	14–6.34E + 03	Cell communication/signal transduction
56	TMEM41A	3q27.2	0.000265	371	15.4–8.90E + 03	Biological process unknown
57	MBNL3	Xq26.2	0.000267	2060	34–1.24E + 05	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
58	WNK3	Xp11.22	0.000272	2.74E + 05	324–2.32E + 08	Biological process unknown
59	KRT3	12q13.13	0.000286	416	16–1.08E + 06	Cell growth and/or maintenance
60	C10orf132	10q24.2	0.000293	3.68	1.82–7.46	Biological process unknown
61	CHRNA5	15q24	0.000298	8150	61.9–1.07E + 06	Cell communication/signal transduction

TABLE 2. (Continued)

No.	Gene	Location	P-value	Hazard ratio	CI of HR	Biological process
62	ONECUT1	15q21.3	0.000302	775	21–2.86E + 04	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
63	FKBPL	6q21.3	0.000314	48.8	5.89–404	Cell communication/signal transduction
64	CYB5R3	22q13.2	0.000317	2.06E-02	2.49E-02–0.17	Metabolism:Energy pathway
65	PRCC	1q21.1	0.000327	52.8	6.07–460	Cell communication/signal transduction
66	FLJ12949		0.000327	141	9.48–2.10E + 03	
67	PCDHGA1	5q31	0.000328	3.02E + 06	879–1.04E + 10	Biological process unknown
68	SH2D6	2p11.2	0.000335	1.97E + 07	2037–1.91E + 11	Cell communication/signal transduction
69	CBY1	22q12	0.000337	8.61E-02	6.40E-04–0.116	Regulation of signal transduction
70	CNDP2	18q22.3	0.000354	4.43E-02	8.00E-03–0.245	Protein metabolism
71	C1orf65	1q41	0.000356	4.88E + 17	9.53E+07–2.50E + 27	Biological process unknown
72	SLC25A37	8q21.2	0.000367	773	19.9–3.00E + 04	Transport
73	MTMR7	8q22	0.000370	3.53	1.766–7.07	Cell communication/signal transduction
74	DRAM	12q23.2	0.000388	2.68E-01	6.25E-02–0.45	Apoptosis
75	LBXCOR1	15q23	0.000401	3.61E + 23	3.26E + 10–3.99E + 36	
76	LOC653216		0.000430	8.85E + 07	3.33E + 04–2.35E + 12	
77	IL31RA	5q11.2	0.000437	8770	55.7–1.38E + 06	Cell communication/signal transduction
78	SH3BP2	4p16.3	0.000439	2.70E + 09	1.49E + 04–4.89E + 14	Cell communication/signal transduction
79	CACNA2D2	3q21.3	0.000458	8.11	2.51–26.1	Transport

DISCUSSION

In this study, we analyzed the relationship between the expression profile pattern and prognosis of EC, simultaneously in both cancer cells and associated stroma, and established a formula based on the gene expression in both cancer and stroma that can, with moderate accuracy, predict the recurrence of EC. Similar to our results, there are previous studies that have demonstrated that stromal gene expression patterns are related to prognosis in breast cancer, ovarian cancer, and prostate cancer (Finak et al., 2008; Farmer et al., 2009; Gregg et al., 2010; Kataoka et al., 2012). Farmer et al. (2009) extracted 50 stromal metagenes by performing microarray analysis, and reported that while these stromal metagenes were unrelated to survival in untreated subjects, they could predict the relapse-free survival of subjects who received anthracycline-based chemotherapy. Finak et al. (2008) established a new SDPP consisting of 26 genes, and demonstrated that SDPP could predict clinical outcomes

in breast cancer. However, there were no overlapping stromal genes between these two reports and our study. In our study, extraction of stromal genes significantly correlated with PFS (Pvalue < 0.05) resulted in 1,593 candidate genes. However, there were only seven genes (*CIQTNF3*, *WNT1*, *TGF-β*, *GZMA*, *HRSLS*, *VGLL1*, and *ADM*) that overlapped with the previous two reports. This may indicate that the stromal reaction is related with the origin of cancer, or the affected organ. Interestingly, Finak et al. (2008) moreover reported that the SDPP predicts outcomes in several published whole tumor-derived expression datasets (Netherlands Cancer Institute, John Radcliffe Hospital, Uppsala, and Rotterdam) and that SDPP has a higher accuracy than all other signatures and predictors in the Netherlands Cancer Institute dataset, including the FDA-approved 70-gene predictor (whole tumor-derived expression data; Finak et al., 2008). However, in this study, the accuracy tended to be higher in cancer cells compared with in stroma.

TABLE 3. The Top 79 Genes Related with Recurrence (Stromal)

No.	Gene	Location	P-value	Hazard ratio	CL of HR	Biological process
1	SPC25	2q31.1	0.000006	15.5	4.74–50.5	Apoptosis
2	KIAA0514	10q11.22	0.000006	16.4	4.88–55.4	Biological process unknown
3	LIN28	1p36.11	0.000010	201	19.2–2110	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
4	SPON2	4p16.3	0.000011	0.137	5.63E-02–0.332	Cell growth and/or maintenance
5	GPC5	13q32	0.000013	2600	75.6–89500	Cell communication/signal transduction
6	FLJ35880		0.000014	3.86E + 15	3.65E + 08–4.10E + 22	
7	KIAA1324	1p13.3	0.000015	0.349	2.16E-01–0.562	Biological process unknown
8	CT45-4	Xq26.3	0.000018	36.8	7.09–191	Biological process unknown
9	PTGER1	19p13.1	0.000021	8.19	3.11–21.6	Cell communication/signal transduction
10	LOC441294		0.000036	6.3	2.63–15.1	
11	CYP1A2	15q24.1	0.000049	27.5	5.55–136	Metabolism:Energy Pathway
12	PCDHGA1	5q31	0.000060	1.02E + 07	3850–2.70E + 10	Biological process unknown
13	SPATC1	8q24.3	0.000075	6.01E + 47	1.35E + 24–2.68E + 71	Biological process unknown
14	WDR70	5q13.2	0.000080	108	10.6–1110	Biological process unknown
15	C17orf55	17q25.3	0.000082	14.6	3.85–55.4	Biological process unknown
16	FBXW12	3p21.31	0.000104	1.59E + 04	120–2.11E + 06	Protein metabolism
17	WDR40B	Xq25	0.000110	745	26.1–2.13E + 04	Cell communication/signal transduction
18	ZNF620	3p22.1	0.000134	5.97E + 27	3.31E + 13–1.08E + 42	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
19	SUSD4	1q41	0.000145	4.17E + 06	1600–1.09E + 10	Immuno response
20	PRCC	1q21.1	0.000148	860	26.2–2.82E + 04	Cell communication/signal transduction
21	ACRV1	11q24.2	0.000163	21.4	4.36–105	Cell communication/signal transduction
22	INTS9	8p21.1	0.000165	0.116	3.80E-02–0.356	Biological process unknown
23	SLC6A10P	16p11.2	0.000168	5.05	2.17–11.8	
24	ADORA3	1p13.2	0.000180	1.32E + 08	7450–2.35E + 12	Cell communication/signal transduction
25	CXCL6	4q13.3	0.000183	981	26.6–36200	Immuno response
26	PSG2	19q13.1–q13.2	0.000184	1.01E + 35	4.60E + 16–2.24E + 53	Immuno response
27	RASAL2	1q24	0.000185	2.48E + 04	123–5.00E + 06	Cell communication/signal transduction
28	TRIO	5q15.2	0.000185	15.2	3.65–63.2	Cell communication/signal transduction
29	ZBTB12	6p21.33	0.000190	331	15.7–6960	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism

TABLE 3. (Continued)

No.	Gene	Location	P-value	Hazard ratio	CL of HR	Biological process
30	ADRB2	5q31-q32	0.000195	0.107	3.31E-02-0.347	Cell communication/signal transduction
31	RALYL	8q21.2	0.000217	3.41	1.78-6.54	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
32	GPRIN1	5q35.2	0.000235	31.5	5.01-198	Biological process unknown
33	FGD4	12p11.21	0.000247	389	16-9440	Cell communication/signal transduction
34	APCDD1L	20q13.32	0.000252	3.91	1.89-8.14	Biological process unknown
35	S100A1	1q21	0.000255	4.41	1.99-9.76	Cell communication/signal transduction
36	CLDN6	16p13.3	0.000260	3.39	1.76-6.52	Cell growth and/or maintenance
37	C10orf91	10q26.3	0.000261	2.23E + 22	2.25E + 10-2.22E + 34	
38	C5orf34	5p12	0.000266	6.17E + 28	2.07E + 15-1.84E + 44	Biological process unknown
39	PTPRT	20q12-q13	0.000273	29.9	4.8-186	Cell communication/signal transduction
40	ISYNA1	19p13.11	0.000294	1340	27.2-66000	Metabolism:Energy Pathway
41	IL1RAPL2	Xq22	0.000299	228	12-4310	Cell communication/signal transduction
42	PRKCBI	16p11.2	0.000305	0.118	3.70E-02-0.377	signal transduction
43	SFRP2	4q31.3	0.000309	7.49	2.51-22.4	Cell communication/signal transduction
44	CAPG	4p15.33	0.000335	2.47E + 06	793-7.67E + 09	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
45	MPP6	7p15	0.000340	6.12	2.27-16.5	Cell communication/signal transduction
46	PDE6B	4p16.3	0.000355	3540	40-3.14E + 05	Metabolism:Energy Pathway
47	FBXW4	10q24	0.000357	5.89E-03	3.52E-04-9.87E-02	Protein metabolism
48	SYT13	11p12-p11	0.000370	3.55	1.77-7.14	Transport
49	FLJ32011		0.000389	16.7	3.53-79.3	
50	HIST1H3G	6p22.1	0.000406	14.3	3.28-62.6	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
51	HUNK	21q22.1	0.000409	343	13.5-8730	Cell communication/signal transduction
52	LOC389257		0.000413	2.60E + 08	5550-1.21E + 13	
53	C13orf35	13q34	0.000449	3.52E + 43	1.68E + 19-7.38E + 67	Biological process unknown
54	KIAA2018	3q13.2	0.000456	3.91E + 09	16900-9.04E + 14	Transcription
55	COL9A3	20q13.3	0.000458	4.24E + 03	39.7-4.53E + 05	Cell growth and/or maintenance
56	B4GALT3	1q21-q23	0.000460	25.4	4.16-155	Metabolism:Energy Pathway
57	UBXN2A	2q23.3	0.000482	17.9	3.54-90.5	Biological process unknown

TABLE 3. (Continued)

No.	Gene	Location	P-value	Hazard ratio	CL of HR	Biological process
58	LHX4	1q25.2	0.000518	6.10E + 03	44.4–8.36E + 05	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
59	PCDHBI	5q31	0.000592	1.47E + 03	22.9–9.41E + 04	Cell growth and/or maintenance
60	HMGA2	12q15	0.000670	2.94E + 04	78.3–1.11E + 07	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
61	NAT12	14q22.3	0.000715	1.59E + 04	58.6–4.31E + 06	Metabolism:Energy Pathway
62	GNBI	1p36.33	0.000724	6.31E –02	1.27E-04–0.313	Cell communication/signal transduction
63	KENAE	5q13.2	0.000768	1.30E + 04	52.2–3.26E + 06	protein metabolism
64	PRR17	20q13.33	0.000771	356	11.6–1.10E + 04	Biological process unknown
65	XIRP2	2q24.3	0.000803	28.7	4.03–204	Cytoskeletal anchoring
66	SFXN1	5	0.000815	7.09	2.25–22.3	Transport
67	PRPF8	17p13.3	0.000837	1.23E-02	9.34E-04–0.163	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
68	HIST1H1D	6p21.33	0.000844	8820	42.5–1.83E + 06	Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism
69	MGAT4A	2q12	0.000870	9.77	2.55–37.4	Metabolism:Energy Pathway
70	OR8G1	11q24.2	0.000880	3.22E + 05	183–5.67E + 08	Cell communication/signal transduction
71	MGC10997		0.000927	5.84	2.06–16.6	
72	MURC	9q31.1	0.000935	315	10.4–9510	Biological process unknown
73	LECT2	5q31.1	0.000948	5.75E + 06	564–5.85E + 10	Immuno response
74	BPY2B	Yq11.223	0.000977	5.52E-34	9.37E-54–3.25E-14	Cell growth and/or maintenance
75	ARHGAP4	Xq28	0.000980	61.7	5.32–716	Cell communication/signal transduction
76	CI6orf73	16p13.3	0.000981	7.71	2.29–26	Biological process unknown
77	AVP	20p13	0.000984	39.5	4.44–352	Reglation of biological process
78	ADCY4	14q12	0.000988	9.30E-02	2.26E-04–0.382	Cell communication/signal transduction
79	MFI2	3q28-q29	0.000997	2.11E + 08	2330–1.91E + 13	Immuno response

We found 17 genes that were included among the top 79 genes of both cancer and stroma. Of these, 10 genes were related to cell communication/signal transduction. We have previously reported that EGR1 expression, not only in cancer but also in stromal cells, is related to prognosis of ovarian cancer, and in agreement with this,

we found that EGR1 was found in the nuclei of cancer cells, as well as in the associated fibroblasts, in EC (Kataoka et al., 2012). There are several interpretations of this phenomenon: (1) these 10 gene products were produced by the cancer cells, and cancer cells stimulate stromal cells, including cancer-associated fibroblasts (CAFs), to

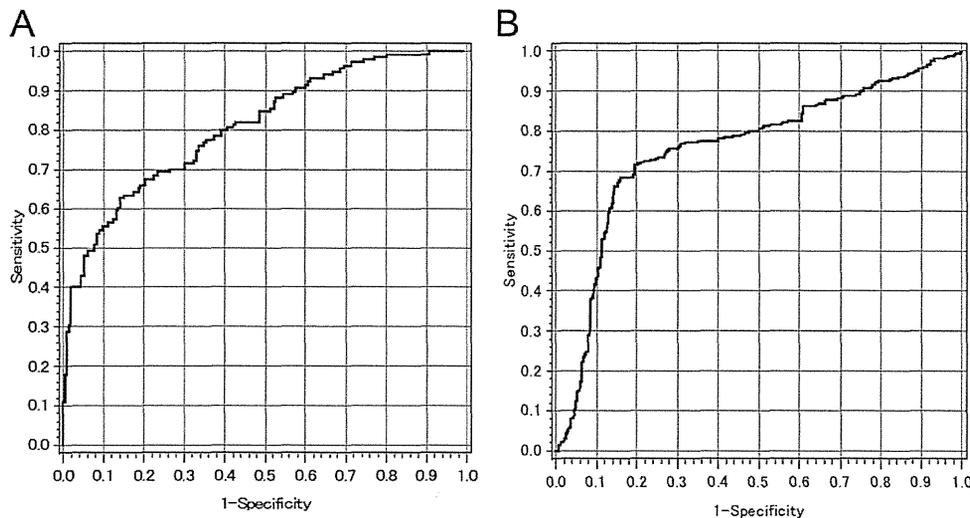


Figure 1. (A) The receiver operating characteristic (ROC) curve with 0.632 adjustment for cancer. The estimated AUC of ROC was 0.800. (B) The ROC curve with 0.632 adjustment for stroma. Estimated AUC of ROC was 0.758.

subsequently produce these proteins; (2) these 10 gene products were produced by stromal cells, including CAFs, and the stromal cells stimulate cancer cells to produce these proteins; and (3) some part of the cancer stroma originated from cancer cells.

Kato et al. (2010) recently reported on the isolation and characterization of side-population (SP) cells in human EC, and demonstrated that SP cells formed large, invasive tumors, comprised of both tumor and stromal-like cells, where the stromal-like cells were derived from the inoculated tumor cells. Cancer-induced fibroblasts are generally thought to be either host cells, including tissue and bone marrow-derived fibroblasts (Hanahan and Weinberg, 2000; Bissell and Radisky, 2001), or alternatively originate from the epithelial mesenchymal transition of cancer cells. Further studies were required to clarify this.

Dakhova et al. (2009) reported that genes encoding growth factors or receptors (ERBB2, FGF19, FLRT1, NOTCH1, WNT10 β , TGFBR2, KIT, EDNRB, and GDNF), extracellular matrix proteins (COMP, MEPE, LAMA2, and FGA), and matrix interacting proteins (SDC4, LRP1, CD36, and THBS4) were upregulated in reactive stroma. Finak et al. (2008) reported that in breast cancer patients with poor prognosis, genes related to angiogenesis, hypoxia, or tumor-associated macrophage responses, such as *ADM*, *IL8*, *EDN1*, *SPP1*, and *CXCL1* were upregulated, whereas genes related to the negative regulation of the WNT receptor (*FRZB*) and development (*HOXA10*) were downregulated. In contrast, in breast cancer

patients with good prognosis, genes associated with the T helper type 1 immune response, including T cell receptor complex (*CD8A*, *CD247*, and *CD3D*), MHC class I protein binding (*CD8A*), and granzyme A and granzyme B activity (*GZMA*) were upregulated. In this study, genes in stroma encoding cell communication/signal transduction proteins (*GPC5*, *PTGER1*, *WDR40B*, *PRCC*, *ACRV1*, *ADORA3*, *RASAL2*, *TRIO*, *ADRB2*, *FGD4*, *S100A1*, *PTPRT*, *IL1RAPL2*, *SFRP2*, *MPP6*, *HUNK*, *GNB1*, *OR8G1*, *ARHGAP4*, and *ADCY4*) or regulation of nucleobase, nucleoside, nucleotide, and nucleic acid metabolism-related proteins (*LIN28*, *ZNF620*, *ZBTB12*, *RALYL*, *CAPG*, *HIST1H3G*, *LHX4*, *HMGA2*, *PRPF8*, and *HIST1H1D*) were common. Immune response-related genes (*SUSD4*, *CXCL6*, *PSG2*, *LECT2*, and *MFI2*) were more common in stroma compared with cancer, whereas apoptosis-related genes (*SPC25*, *GIMAP5*, *ZNF382*, and *DRAM*) were more common in cancer compared with stroma.

Based on the 79 genes of cancer, the 22 recurrent cases were divided into two groups, excluding three cases. One group tended to include grade three tumors. However, based on the 79 genes of stroma, the 22 recurrent cases were almost homogeneous, excluding three cases. This may indicate that the recurrence-related genes in cancer differ between high-grade and low-grade tumors. In contrast, stromal gene expression is likely not dependent on the grade of cancer. It is generally thought to be difficult to find unique biomarkers unrelated to histological type as cancer usually consists of

various types of histology. Stromal-based biomarkers may solve this problem.

In conclusion, the formula based on the gene expression profile of cancer and stroma established here can predict the recurrence of EC. The recurrence-related genes in cancer differed between high-grade and low-grade tumors, whereas stromal gene expression was not dependent on the tumor grade.

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