

A novel gene expression scoring system for accurate diagnosis of basaloid squamous cell carcinoma of the esophagus

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Abstract. Basaloid squamous cell carcinoma of the esophagus (BSCE) is a rare variant of squamous cell carcinoma that is difficult to distinguish from other carcinomas by preoperative endoscopic biopsy because of its histological varieties. Accurate diagnosis is essential for adequate treatment, and the methods proposed so far (e.g., immunohistochemical staining) have limitations. In this study, we tried to identify the characteristic bundles of gene expression in BSCE using comprehensive gene expression analysis (CGEA). Subsequently, we constructed a gene expression scoring system for the proper diagnosis of BSCE. Fifty-seven surgical specimens, including seven BSCEs, obtained from 30 patients who underwent esophagectomy were used for constructing the scoring system. Three hundred and twelve biopsy specimens, including eight BSCEs, obtained from 80 patients and 20 commercially available formalin-fixed paraffin-embedded (FFPE) specimens diagnosed as esophageal cancer, including 13 BSCEs, were used for validation. After our original mathematical extraction algorithm, 75 genes were extracted to distinguish BSCE from non-BSCE. The cumulative converted values (gene expression score) of the respective 75 genes

from each specimen were obtained and lined up in ascending order to assess the optimal gene expression cut-off score for a definitive diagnosis of BSCE. The validation of this scoring system showed high prediction of the biopsy specimens [area under the curve (AUC)=0.981; 95% confidence interval (CI): 0.952-1.000] and the commercially available FFPE specimens (AUC=0.901; 95% CI: 0.750-1.000). In conclusion, using CGEA in a gene expression scoring system helps in differentiating BSCE from non-BSCE with high accuracy and may contribute in improving BSCE treatment.

Introduction

Basaloid squamous cell carcinoma (BSC) was first reported by Wain *et al* (1) to occur in the head and neck region. BSC may occur in various other sites, including esophagus (2), lung (3), anus (4), uterine cervix (5), penis (6), and urinary bladder (7). BSC of the esophagus (BSCE) is a rare and uncommon variant of squamous cell carcinoma (SCC), with a reported incidence ranging from 1.0 to 8.7% (8-16) in Japan and 0.4 to 11.3% (2,17-24) in other countries.

BSCE has six typical components on histology: solid nest with central necrosis, cribriform pattern, ductal differentiation, microcyst and/or trabecular nests, hyaline-like material deposition, and coexistence of SCC components (16). Because of these histological varieties, BSCE is difficult to distinguish from adenoid cystic carcinoma, small cell carcinoma, poorly differentiated SCC, or adenosquamous carcinoma (2,23,25,26). Furthermore, it is even more difficult to diagnose BSCE based on the histological examination of endoscopic biopsy specimens, with a low diagnostic accuracy of only 0-10% (23,24,27). BSCE has been frequently diagnosed as SCC on endoscopic biopsy specimens, probably because of the fact that BSCE frequently presents as a submucosal tumor-like structure covered with normal epithelium or SCC (14,15). Therefore, sampling multiple and deeper sites is recommended for diagnosis by endoscopic biopsy (24,27). Some diagnostic approaches using immunoreactivity (2,9,12,16,18,19) or polymerase chain reaction (PCR) (28,29) have been reported, but none of these showed high specificity for BSCE.

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Abbreviations: BSC, basaloid squamous cell carcinoma; BSCE, BSC of the esophagus; CGEA, comprehensive gene expression analysis; FFPE, formalin-fixed paraffin-embedded; AUC, area under the curve; CI, confidence interval; SCC, squamous cell carcinoma; PCR, polymerase chain reaction; NEC, neuroendocrine carcinoma; CK, cytokeratin; SD, standard deviation; ROC, receiver operating characteristic

Key words: basaloid squamous cell carcinoma, comprehensive gene expression analysis, diagnostic method, gene expression scoring system, biopsy specimens

The prognosis of BSCE is still controversial. Some studies stated no significant difference between BSCE and SCC (2,20), whereas others stated poorer prognosis of BSCE than that of SCC (16,17,23). Some authors specified that BSCE shows a poor degree of differentiation, high proliferative activity (2), aggressive biological behavior (16), high telomerase activity (20), and a worse prognosis than that in SCC in advanced cases (30). Meanwhile, other authors mentioned that the treatment for BSCE is similar to that for SCC of the esophagus (15,23). The rarity of and difficulty in the proper diagnosis of BSCE (15) may be responsible for this diversity. Therefore, proper diagnosis is mandatory for analyzing the outcome and determining the suitable treatment for this disease entity.

We have previously reported the use of comprehensive gene expression analysis (CGEA) to identify some disease-specific genes (31,32). The present study aimed to improve the diagnostic accuracy for BSCE by attempting to extract the genes expressed in it. From CGEA of esophagectomy specimens, we constructed, verified, and evaluated a gene expression scoring system for the proper diagnosis of BSCE.

Materials and methods

Patient selection. We initially enrolled all 113 esophageal cancer patients who underwent esophagectomy and/or endoscopic biopsy at Fukushima Medical University Hospital from January 2008 to July 2015. Among these patients, 14 patients (1 in stage 0, 4 in stage II, 4 in stage III, 4 in stage IVa, 1 in stage IVb and 1 in unknown stage) were not followed in our department, and one surviving patient (stage II) denied to participate in research. These cases were excluded.

Ethics statement. This study was approved by the ethics committee of Fukushima Medical University (approval no. 1953). Written informed consent was obtained from 98 patients.

Commercially available esophageal specimens. From US Biomax Inc. (Rockville, MD, USA), we purchased 20 formalin-fixed paraffin-embedded (FFPE) specimens that were diagnosed to have BSCE components. These FFPE specimens were reviewed by three pathologists before inclusion.

Specimen sampling. Small fractions (7x7 mm) of the cancerous site and normal mucosa were removed from each surgical specimen and were immediately frozen in liquid nitrogen before performing CGEA. Residual tissue specimens were fixed in formalin and then embedded in paraffin before pathological examination.

For the biopsy specimens, tiny fractions (3x3 mm) of the esophageal epithelium, including cancerous and normal sites, were obtained endoscopically; they were immediately frozen separately in liquid nitrogen before performing CGEA. Another specimen from near the first biopsy site was obtained endoscopically; it was fixed in formalin and embedded in paraffin before pathological examination. We made an effort to sample multiple and deeper sites by endoscopic biopsy. We ascertained that the frozen specimens for CGEA and the FFPE specimens for pathological examination had identifiable pathological features.

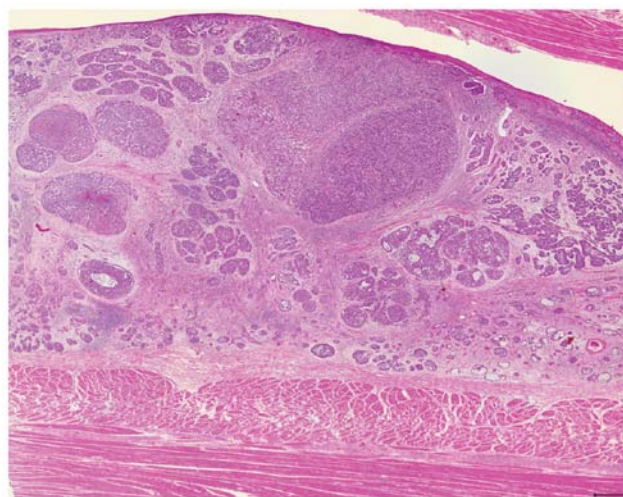


Figure 1. Image of a representative H&E staining in case 4.

Table I. Sources of specimens for CGEA.

Histology	Surgical specimens (N=57)	Biopsy specimens (N=312)	Commercially available
			FFPE specimens (N=20)
Normal esophageal tissue	26	229	0
SCC	23	51	2
BSCE	7	8	13
NEC	1	1	5
Adenocarcinoma	0	21	0
Intraepithelial neoplasia	0	2	0

CGEA, comprehensive gene expression analysis; FFPE, formalin-fixed paraffin-embedded; SCC, squamous cell carcinoma; BSCE, basaloid squamous cell carcinoma of the esophagus; NEC, neuroendocrine carcinoma.

Pathological review. The surgical, endoscopic biopsy and commercially available FFPE specimens were stained with hematoxylin and eosin and were reviewed by three pathologists (Fig. 1). BSCE was defined using the criteria described by Wain *et al.* (1); the six component histological features reported by Imamhasan *et al.* (16) were also evaluated. In this study, tumors that contained some BSC components within SCC were categorized as BSCE.

Comprehensive gene expression analysis. Frozen specimens were processed for total RNA extraction using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan) and for poly(A)+RNA purification using MicroPoly(A) Purist kit (Ambion, Austin, TX, USA). Commercially available FFPE specimens were processed for total RNA extraction using ISOGEN PB kit (Nippon Gene Co., Ltd.). The human common reference RNA was prepared by mixing equal amounts of total RNA and poly(A)+RNA, which were extracted from 22 human cancer cell lines (A431, A549, AKI, HBL-100, HeLa, HepG2, HL60, IMR-32, Jurket,

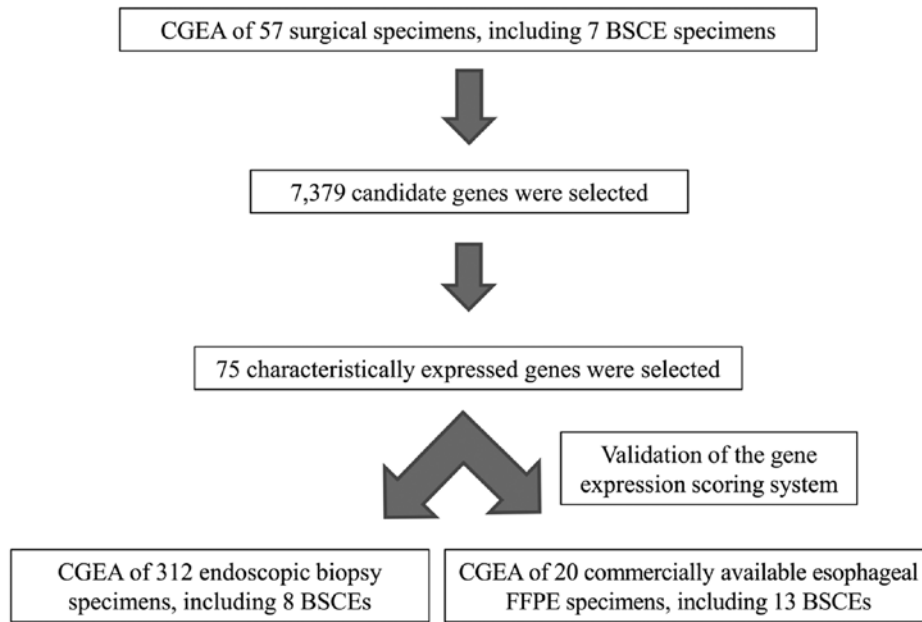


Figure 2. Study design. CGEA, comprehensive gene expression analysis; BSCE, basaloid squamous cell carcinoma of the esophagus; FFPE, formalin-fixed paraffin-embedded.

K562, KP4, MKN7, NK-92, Raji, RD, Saos-2, SK-N-MC, SW-13, T24, U251, U937, and Y79).

The DNA microarray that used poly(A)+RNA was named system 1; a set of synthetic polynucleotides (80-mers) representing 31,797 species of human transcript sequences was printed on a glass slide using a custom arrayer. The DNA microarray that used total RNA was named system 2; a set of synthetic polynucleotides (80-mers) representing 14,400 species of human transcript sequences was printed on a glass slide using a custom arrayer. For RNA of the samples, SuperScript II (Invitrogen Life Technologies, Carlsbad, CA, USA) and Cyanine 5-dUTP (Perkin-Elmer Inc., Boston, MA, USA) were used to synthesize labeled cDNA from 2 μ g of poly(A)+RNA in system 1 and 5 μ g of total RNA in system 2. Using the same method for the reference RNA, Cyanine 3-dUTP (Perkin-Elmer Inc.) was used to synthesize labeled cDNA from 2 μ g of poly(A)+RNA in system 1 and 5 μ g of total RNA in system 2.

Hybridization was performed with a Labeling and Hybridization kit (MicroDiagnostic, Tokyo, Japan). Signals were measured using a GenePix 4000B Scanner (Axon Instruments, Inc., Union City, CA, USA) and then processed into the primary expression ratios of the cyanine 5 intensity of each specimen to the cyanine 3 intensity of the human common reference RNA. Each ratio was normalized using GenePix Pro 3.0 software (Axon Instruments, Inc.). The primary expression ratios were converted into log₂ values, which were designated as log ratios or converted value. Data were processed using Microsoft Excel software (Microsoft, Bellevue, WA, USA) and MDI gene expression analysis software package (MicroDiagnostic) (33).

Statistical analysis. Clustering analysis was performed using group average method with an Expression View Pro (MicroDiagnostic).

The cut-off score was determined by receiver operating characteristic (ROC) curve analysis with the aim of validating the gene scoring system. The optimal cut-off score for the definitive diagnosis of BSCE was assessed and determined by area under the ROC curve (AUC) analysis of the maximum values of sensitivity and specificity. ROC curve analysis was performed using the software program SPSS version 23 (SPSS, Inc., Chicago, IL, USA).

Refinement steps to identify candidate genes from CGEA of surgical specimens. Step 1: Genes with fluorescence intensity below the detection limit in two or more of the seven BSCE specimens were excluded. Step 2: The genes with a converted value of ≥ 1 in at least one of the 57 surgical specimens were selected. Step 3: The mean or average of the converted values of the chosen genes were calculated, and the genes that met the following requirement were selected: average value - converted value ≥ 1 . Step 4: Clustering analysis was performed on the chosen genes.

Construction of gene expression scoring system for BSCE. Step 5: The mean of the converted values of the genes that were expressed in six specimens of the BSCE cluster was calculated; genes with an average value of ≥ 1 were selected. Step 6: Genes with fluorescence intensity below the detection limit were excluded in more than half of the non-BSCE specimens. Step 7: The standard deviation (SD) of the converted values of the genes that were expressed in the non-BSCE specimens were calculated; genes with an SD of < 0.5 were selected. Step 8: Genes that met the following requirement were selected: average value of six specimens in the BSCE cluster - average value of non-BSCE specimens of ≥ 1 . Step 9: A t-test was used to compare the average value of six specimens between the BSCE cluster and the non-BSCE specimens; genes with a P-value of < 0.01 were selected.

Table II. Clinicopathologic features of six esophagectomy cases.

Age, sex	Biopsy diagnosis	Pathologic diagnosis	Tumor size (mm)	Tumor type	Depth of invasion (pT)	Lymph node metastasis (pN)	Lymphatic invasion (ly)	Venous invasion (v)	UICC stage	Prognosis
1 68, M	ASC	BSCE	42	Type 2	T1	N0	0	1	IA	58 months-died (lung metastasis)
2 55, M	SCC	BSCE	30	Type 5	T1	N0	0	2	IA	60 months-alive
3 56, M	SCC	BSCE	25	Type 3	T4	N3	2	2	IIIC	19 months-died (lung metastasis)
4 68, F	SCC (first), BSCE (second)	BSCE	18	Type 2	T1	N0	0	1	IA	37 months-alive
5 64, M	BSCE	BSCE	12	Type 0-IIa	T1	N1	0	0	IIB	29 months-alive (LN metastasis)
6 65, M	BSCE	BSCE, SCC	5, 45	Type 0-IIc	T1	N0	0	0	IA	7 months-alive

UICC, Union for International Cancer Control; ASC, adenosquamous carcinoma; SCC, squamous cell carcinoma; BSCE, basaloid squamous cell carcinoma; LN, lymph node.

Step 10: The converted values of the selected genes from all specimens were added as gene expression scores, which were arranged in ascending order.

Study design. First, using CGEA of the surgical specimens, genes that were characteristically expressed in BSCE were identified; subsequently, a gene expression scoring system was constructed to more accurately diagnose BSCE. Second, the accuracy of our scoring system was validated using biopsy and commercially available FFPE specimens. The study design is shown in Fig. 2.

Results

Pathological diagnosis of the specimens for CGEA. Of the 98 patients, surgical specimens were obtained from 30, endoscopic biopsy specimens from 80, and both from 12. Seven cases of BSCE were included in this study, six cases underwent esophagectomy and one case underwent only endoscopic biopsy. We obtained more than one specimen from each individual, and all specimens were subjected to gene expression analysis. The number of specimens that contained enough amount of RNA for CGEA was 369 (57 surgical and 312 endoscopic) (Table I). The surgical specimens comprised 26 normal esophageal tissues, 23 SCCs, seven BSCEs, and one neuroendocrine carcinoma (NEC). Biopsy specimens comprised 229 normal esophageal tissues, 51 SCCs, eight BSCEs, one NEC, 21 adenocarcinomas, and two intraepithelial neoplasias. Commercially available FFPE specimens comprised two SCCs, 13 BSCEs, and five NECs. All commercially available FFPE specimens were also subjected to CGEA.

Clinicopathological characteristics. Patients with BSCE, including five men and one woman, with a mean age of 63 (range, 55-68) years, underwent esophagectomy; their clinicopathological characteristics are listed in Table II. Only three of six patients (50%) were diagnosed as having BSCE by preoperative endoscopic biopsy. The mean tumor size

was 28.6 (range, 12-45) mm. Based on the seventh Union for International Cancer Control tumor-node-metastasis classification of malignant tumors, the pathological stage was stage I in four patients, stage II in one, and stage III in one. Within a mean follow-up period of 35 (range, 7-60) months, two patients died because of BSCE, one remained alive with recurrence, and three remained alive without recurrence.

Comprehensive gene expression analysis of the surgical specimens. Fig. 3 shows the result of CGEA of 57 surgical specimens, including seven BSCE specimens (one specimen from cases 1, 2, 3, 5 and three specimens from case 4); 10,027 genes were selected in step 1; 9,004 in step 2; and 7,379 in step 3.

A two-dimensional hierarchical clustering analysis of 7,379 genes yielded three different clusters: the 1) BSCE cluster, which comprised six of the seven BSCE specimens; 2) SCC cluster, which mainly comprised SCC; and 3) normal cluster, which mainly comprised normal esophageal tissue. It was possible to distinguish BSCE specimens from the others using this analysis.

Gene expression scoring system for BSCE. We selected BSCE-specific candidate maker genes and attempted to construct a gene expression scoring system for more accurate diagnosis of BSCE, and 986, 972, 243, and 100 genes were sequentially selected from steps 5, 6, 7, and 8, respectively. Finally, 75 genes were selected in step 9 (Table III) and subjected to extrapolation of the gene expression score, as described in step 10. Our gene expression scoring system, which set the cut-off score at 56.5, very clearly distinguished the seven BSCE specimens from the non-BSCE specimens (Fig. 4).

Validation of the gene expression scoring system. Using CGEA, we calculated 75 gene expression scores, which were arranged in ascending order (Fig. 5). ROC curve analysis of the gene expression scoring system using biopsy specimens

Table III. Genes characteristically expressed in BSCE based on CGEA of surgical specimens.

No.	ID	Symbol	Name
1	NM_001033568.2	RHOT1	Ras homolog family member T1 (RHOT1), transcript variant 1
2	NM_015690.4	STK36	Serine/threonine kinase 36 (STK36), transcript variant 1
3	NM_000915.3	OXT	Oxytocin/neurophysin I prepropeptide (OXT)
4	NM_001409.3	MEGF6	Multiple EGF-like-domains 6 (MEGF6)
5	NM_021197.3	WFDC1	WAP four-disulfide core domain 1 (WFDC1)
6	NM_020796.4	SEMA6A	Sema domain, transmembrane domain (TM), cytoplasmic domain, (semaphorin) 6A (SEMA6A)
7	NM_153213.3	ARHGEF19	Rho guanine nucleotide exchange factor (GEF) 19 (ARHGEF19)
8	XM_005261771.3	PLA2G6	Phospholipase A2, group VI (cytosolic, calcium-independent) (PLA2G6), transcript variant X18
9	NM_000933.3	PLCB4	Phospholipase C, β 4 (PLCB4), transcript variant 1
10	NM_023110.2	FGFR1	Fibroblast growth factor receptor 1 (FGFR1), transcript variant 1
11	AK055081.1		cDNA FLJ30519 fis, clone BRAWH2000859
12	NM_000346.3	SOX9	SRY (sex determining region Y)-box 9 (SOX9)
13	NM_025176.4	NINL	Ninein-like (NINL)
14	NM_014698.2	TMEM63A	Transmembrane protein 63A (TMEM63A)
15	NM_020870.3	SH3RF1	SH3 domain containing ring finger 1 (SH3RF1)
16	NM_001110514.1	EBF4	Early B-cell factor 4 (EBF4)
17	NR_036481.2	FGD5P1	FYVE, RhoGEF and PH domain containing 5 pseudogene 1 (FGD5P1), non-coding RNA
18	NM_005117.2	FGF19	Fibroblast growth factor 19 (FGF19)
19	NM_032192.3	PPP1R1B	Protein phosphatase 1, regulatory (inhibitor) subunit 1B (PPP1R1B)
20	NM_020659.3	TTYH1	Tweety family member 1 (TTYH1), transcript variant 1
21	NM_145804.2	ABTB2	Ankyrin repeat and BTB (POZ) domain containing 2 (ABTB2)
22	NM_194302.3	CCDC108	Coiled-coil domain containing 108 (CCDC108), transcript variant 1
23	NM_002995.2	XCL1	Chemokine (C motif) ligand 1 (XCL1)
24	NM_001940.3	ATN1	Atrophin 1 (ATN1), transcript variant 2
25	AK021565.1		cDNA FLJ11503 fis, clone HEMBA1002113
26	NM_006941.3	SOX10	SRY (sex determining region Y)-box 10 (SOX10)
27	NM_003222.3	TFAP2C	Transcription factor AP-2 γ (activating enhancer-binding protein 2 γ) (TFAP2C)
28	NM_003963.2	TM4SF5	Transmembrane 4 L six family member 5 (TM4SF5)
29	NM_002180.2	IGHMBP2	Immunoglobulin mu-binding protein 2 (IGHMBP2)
30	NM_015696.4	GPX7	Glutathione peroxidase 7 (GPX7)
31	NM_017789.4	SEMA4C	Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4C (SEMA4C)
32	NM_178502.3	DTX3	Deltex 3, E3 ubiquitin ligase (DTX3), transcript variant 1
33	NM_014937.3	INPP5F	Inositol polyphosphate-5-phosphatase F (INPP5F), transcript variant 1
34	NM_001380.4	DOCK1	Dedicator of cytokinesis 1 (DOCK1), transcript variant 2
35	NM_007081.2	RABL2B	RAB, member of RAS oncogene family-like 2B (RABL2B), transcript variant 2
36	AK055044.1	TARBP1	TAR (HIV-1) RNA-binding protein 1 (TARBP1)
37	NM_006312.5	NCOR2	Nuclear receptor corepressor 2 (NCOR2), transcript variant 1
38	NM_007270.4	FKBP9	FK506-binding protein 9, 63 kDa (FKBP9), transcript variant 1
39	NM_016162.3	ING4	Inhibitor of growth family, member 4 (ING4), transcript variant 1
40	NM_005937.3	MLLT6	Myeloid/lymphoid or mixed-lineage leukemia; translocated to 6 (MLLT6)
41	AK021700.1		cDNA FLJ11638 fis, clone HEMBA1004323
42	NM_015662.2	IFT172	Intraflagellar transport 172 (IFT172)
43	NM_032501.3	ACSS1	Acyl-CoA synthetase short-chain family member 1 (ACSS1), transcript variant 1
44	NM_016102.3	TRIM17	Tripartite motif containing 17 (TRIM17), transcript variant 1
45	NM_152753.3	SCUBE3	Signal peptide, CUB domain, EGF-like 3 (SCUBE3), transcript variant 1
46	NM_133455.3	EMID1	EMI domain containing 1 (EMID1), transcript variant 1

Table III. Continued.

No.	ID	Symbol	Name
47	NM_014640.4	TTL4	Tubulin tyrosine ligase-like family member 4 (TTL4)
48	NM_001161616.2	RGL3	Ral guanine nucleotide dissociation stimulator-like 3 (RGL3), transcript variant 1
49	NM_024798.2	SNX22	Sorting nexin 22 (SNX22), transcript variant 1
50	NM_032781.3	PTPN5	Protein tyrosine phosphatase, non-receptor type 5 (striatum-enriched) (PTPN5), transcript variant 2
51	NM_005996.3	TBX3	T-box 3 (TBX3), transcript variant 1
52	NM_000875.4	IGF1R	Insulin-like growth factor 1 receptor (IGF1R), transcript variant 1
53	NM_178238.3	PILRB	Paired immunoglobulin-like type 2 receptor β (PILRB)
54	NM_152748.3	KIAA1324L	KIAA1324-like (KIAA1324L), transcript variant 1
55	NM_003505.1	FZD1	Frizzled class receptor 1 (FZD1)
56	NM_173812.4	DPY19L2	Dpy-19-like 2 (<i>C. elegans</i>) (DPY19L2)
57	NM_032447.3	FBN3	Fibrillin 3 (FBN3)
58	NM_001987.4	ETV6	Ets variant 6 (ETV6)
59	NM_017563.3	IL17RD	Interleukin 17 receptor D (IL17RD)
60	NM_032040.4	CCDC8	Coiled-coil domain containing 8 (CCDC8)
61	NM_018257.2	PCMTD2	Protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 2 (PCMTD2), transcript variant 1
62	NM_152730.5	TBC1D32	TBC1 domain family, member 32 (TBC1D32), transcript variant 1
63	NM_152739.3	HOXA9	Homeobox A9 (HOXA9)
64	NM_021156.3	TMX4	Thioredoxin-related transmembrane protein 4 (TMX4)
65	NM_002507.3	NGFR	Nerve growth factor receptor (NGFR)
66	NM_004776.3	B4GALT5	UDP-Gal:betaGlcNAc β 1,4-galactosyltransferase, polypeptide 5 (B4GALT5)
67	NM_015544.2	TMEM98	Transmembrane protein 98 (TMEM98), transcript variant 1
68	NM_001852.3	COL9A2	Collagen, type IX, α 2 (COL9A2)
69	NM_005247.2	FGF3	Fibroblast growth factor 3 (FGF3)
70	NM_002523.2	NPTX2	Neuronal pentraxin II (NPTX2)
71	NM_001853.3	COL9A3	Collagen, type IX, α 3 (COL9A3)
72	NM_001851.4	COL9A1	Collagen, type IX, α 1 (COL9A1), transcript variant 1
73	NM_014289.3	CAPN6	Calpain 6 (CAPN6),
74	NM_002336.2	LRP6	Low-density lipoprotein receptor-related protein 6 (LRP6)
75	NM_001692.3	ATP6V1B1	ATPase, H ⁺ transporting, lysosomal 56/58 kDa, V1 subunit B1 (ATP6V1B1)

yielded an optimal cut-off score of 40.5, with an AUC of 0.981, sensitivity of 87.5%, and specificity of 99.0% (Fig. 6A).

By the same procedure, ROC curve analysis of the gene expression scoring system using commercially available FFPE specimens, including 13 BSCE, yielded an optimal cut-off score of 34.9, with AUC of 0.901, sensitivity of 92.3%, and specificity of 71.4% (Fig. 6B).

Discussion

Using CGEA of esophagectomy specimens, we identified the 75 genes that were characteristically expressed in BSCE to construct a gene expression scoring system, which made it possible to distinguish BSCE from non-BSCE in biopsy and commercially available FFPE specimens with high sensitivity and specificity. To our knowledge, this is the first report to show an accurate diagnostic modality that could significantly contribute in improving the diagnosis and treatment of BSCE.

Diagnosing BSCE using endoscopic biopsy specimens is difficult, with a reported diagnostic accuracy of only 0% to 10% (23,24,27). We too were unable to accurately diagnose BSCE using preoperative endoscopic biopsy in three of six surgical patients. To overcome this difficulty, immunohistochemical staining was performed. Cytokeratin (CK) subtypes, including CK13 (12), CK14 (16), and CK19 (9,12,19), were attempted, but they failed to show specific properties, as did p53 and Rb protein (12,34,35). On the other hand, a combination of immunohistochemical staining and PCR analysis was previously tested for differential diagnosis: Bcl-2 expression together with c-myc amplification was demonstrated to be more frequent in BSCE than in SCC (28), but the specificity of this test was low (43.5%). In this study, we identified the 75 genes that were characteristically expressed in BSCE. None of these genes were mentioned in previous reports. Of 75 genes, collagen related genes (COL9A2, COL9A3, COL9A1) and fibroblast growth factor related genes (FGF19, FGF3)

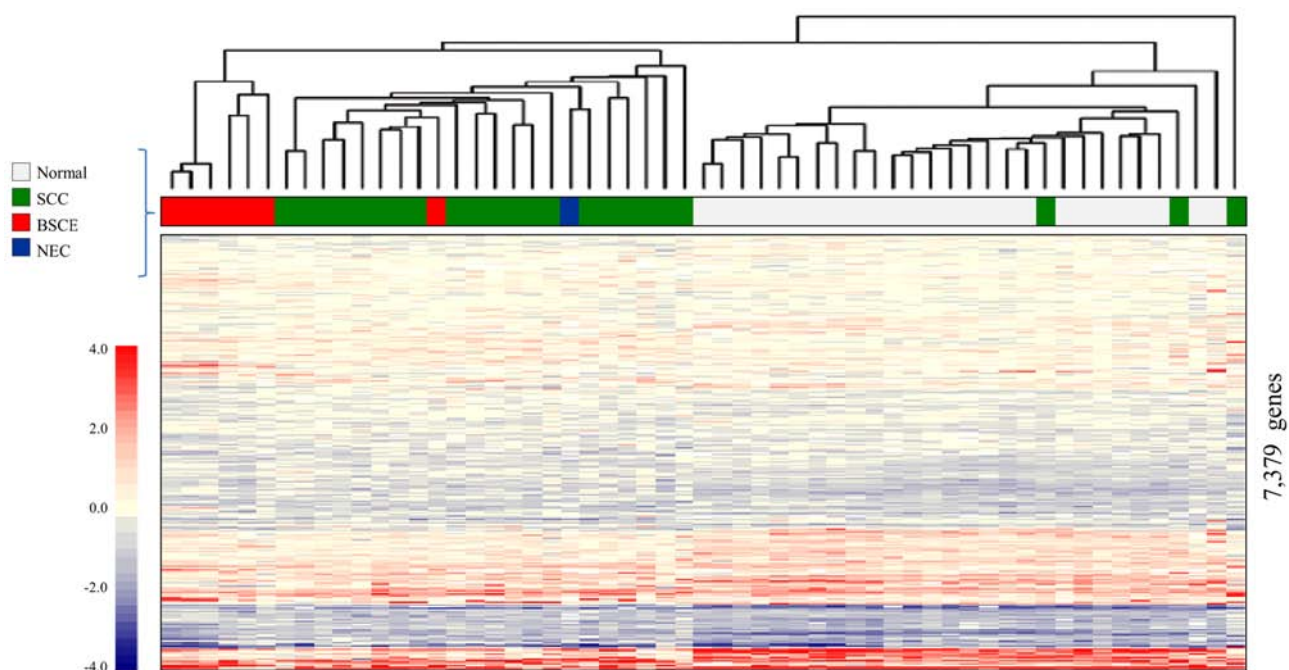


Figure 3. Comprehensive gene expression analysis of 57 surgical specimens. Specimens and genes are aligned in the order defined by the results of the clustering analysis. The dendrogram indicates the relationship among the specimens based on dissimilarity coefficients calculated through clustering analysis. The color bar at the left side of the figure represents the grades of the relative expression levels: increase (red), decrease (blue). SCC, squamous cell carcinoma; BSCE, basaloid squamous cell carcinoma of the esophagus; NEC, neuroendocrine carcinoma.

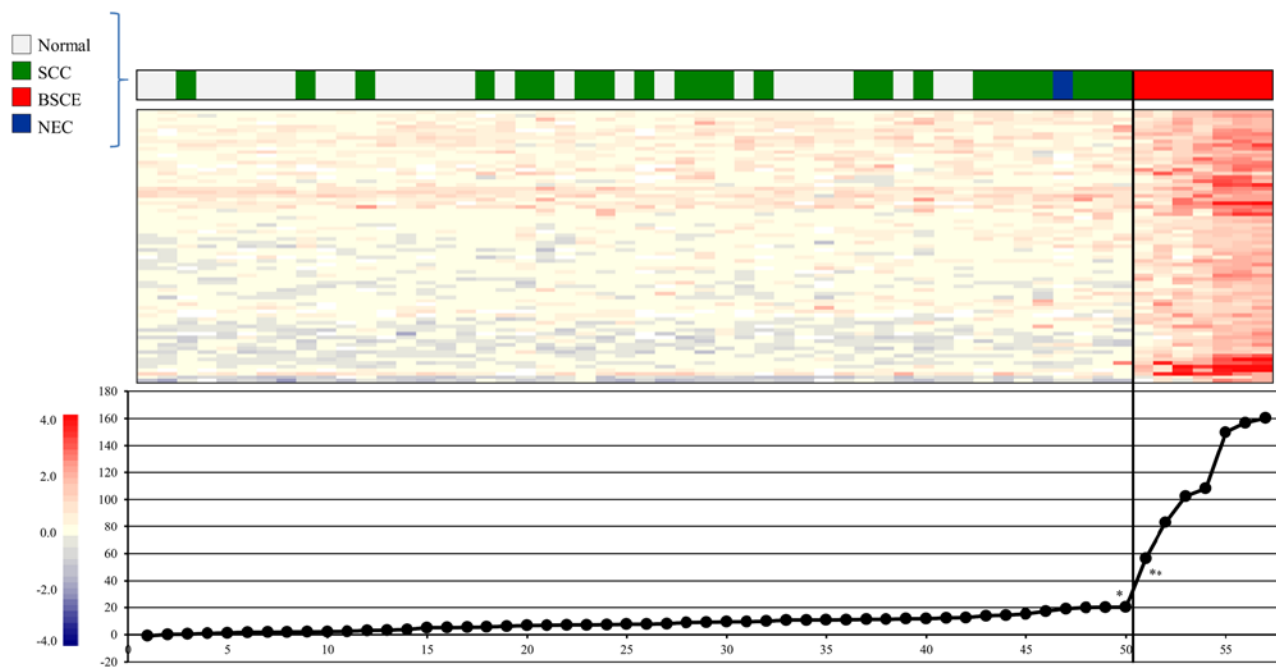


Figure 4. Gene expression scoring system for BSCE using surgical specimens. Line graph depicts the gene expression score. Black vertical line indicates the border between BSCE and non-BSCE. The color bar at the left side of the figure represents the grades of the relative expression levels: increase (red), decrease (blue). X-axis of the graph indicates a number of specimens, and y-axis indicates a gene expression score. *20.2 and **56.5. SCC, squamous cell carcinoma; BSCE, basaloid squamous cell carcinoma of the esophagus; NEC, neuroendocrine carcinoma.

might be concerning the characteristics of BSCE based on the association with genes identified in this study.

In one study, comprehensive gene expression profiling was performed for endoscopic biopsy specimens of esophageal SCC (36). However, CGEA in our institution is different from that in the other institution. We had previously extracted

some disease-specific genes through the CGEA system at our institution (31,32). Our CGEA has three features: 1) it can analyze small samples, like endoscopic biopsy specimens; 2) it can be performed without RNA amplification; and 3) the gene expression ratio of all types of samples can be compared with human common reference RNA. Comprehensive gene

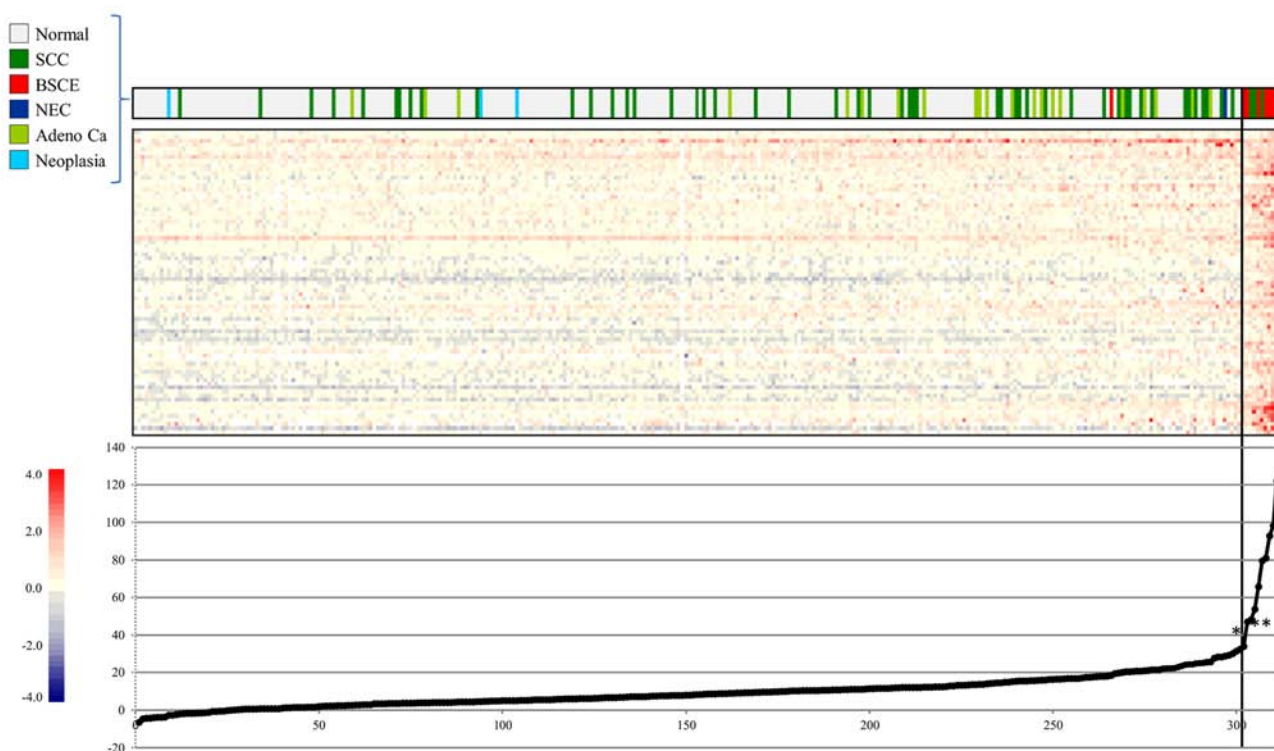


Figure 5. Validation of gene expression scoring system using endoscopic biopsy specimens. Black vertical line indicates the optimal cut-off score through ROC curve analysis. X-axis of the graph indicates a number of specimens, and y-axis indicates a gene expression score. *34.0 and **47.0. ROC, receiver operating characteristic; SCC, squamous cell carcinoma; BSCE, basaloid squamous cell carcinoma of the esophagus; NEC, neuroendocrine carcinoma.

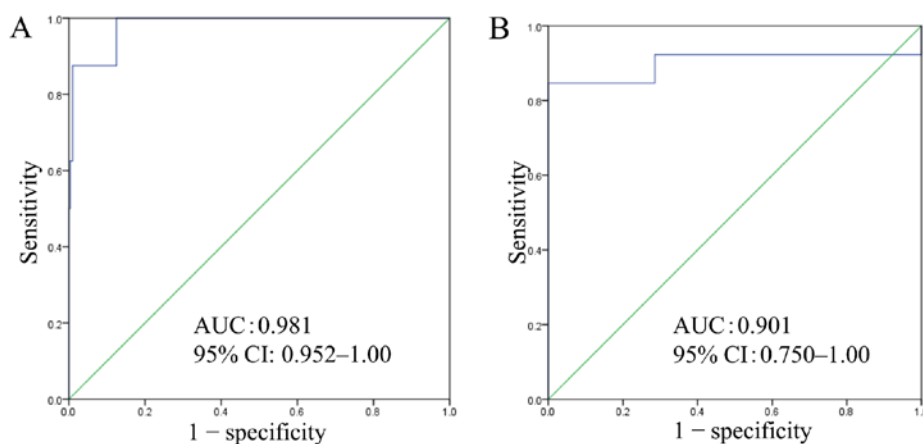


Figure 6. ROC curve analysis. (A) For the endoscopic biopsy specimens, the optimal cut-off score is 40.5. AUC is 0.981, with a sensitivity of 87.5% and a specificity of 99.0%. (B) For the commercially available FFPE specimens, the optimal cut-off score is 34.9. AUC is 0.901, with a sensitivity of 92.3% and a specificity of 71.4%. ROC, receiver operating characteristic; AUC, area under the curve; CI, confidence interval; FFPE, formalin-fixed paraffin-embedded.

expression analysis is done to compare the expression levels of the human common reference RNA which was prepared from 22 human cancer cell lines. Profiles were obtained even from the BSC samples comprising SCC components. A group of genes specific for BSC were selected by comparing BSC containing SCC component with non-BSC (SCC). A group of genes that were expressed in SCC components were eliminated by selection process for the genes specific for BSC. Therefore, the group of genes specific for BSC can distinguish between the BSC and non-BSC even though BSC samples included SCC components. Introducing of the scoring system enabled us to differentiate BSC from non-BSC if the sample contains

a limited portion of BSC with SCC components. In this study, we selected 75 genes for constructing a gene expression scoring system for the proper diagnosis of BSCE. There have been no reports on studies in which a gene expression scoring system was constructed to diagnose cancers. This scoring system is a novel, precise, and powerful tool for diagnosing BSCE in both endoscopic biopsy and FFPE specimens.

There are several limitations in this study. First, the biopsy specimens obtained for histology and CGEA were not identical, although we tried as much as possible to choose specimens that were adjacent to each other. In addition, biopsy samples might not hit the component of BSC for accurate

diagnosis by pathological and genetical analysis. Thus, biopsy samples should be obtained from multiple sites in deep portion of tumor to obtain histological characteristics of BSC. In contrast, with this method, we are able to diagnose BSC from very small amount of specimens as long as it contains BSC component. Second, the parameters of processing the commercially available FFPE specimens (i.e., interval between resection and fixation, duration of fixation) were not controlled. Third, the cut-off scores varied among the sources (surgical, endoscopic biopsy, and FFPE specimens); this may have affected the attainment of reasonable specificity and sensitivity. In this study, the number of patients was too small to enable comparison of the prognoses between BSCE and SCC. These limitations should be addressed using a larger number of cases in the future. Nevertheless, we believe that this method elucidates the proper diagnosis of very rare cases of BSCE. Furthermore, it may be able to clarify whether the prognosis of patients with BSCE is similar to or poorer than that of patients with SCC. Lastly, recently (December 1, 2016) International Cell Line Authentication Committee released Version 8.0 of database of cross-contaminated or misidentified cell lines (37), in which we found five cell lines (AKI human melanoma, HBL-100 human breast carcinoma, human gastric carcinoma MKN-7, SK-N-MC human neuroblastoma and U937 lymphoma histiocytic cells) among our 22 reference cell lines have been contaminated with HeLa cervical adenocarcinoma cells, human cells of unknown origin, a cell line of unknown origin, human Sarcoma (Ewing's) cells and a cell line of unknown origin, respectively. Even with this condition of reference cell lines it is obvious that our results would not be affected since we only used a relative ratio of BSCE against SCC, but not an absolute ratio to reference cell lines in order to select the responsible genes for the scoring system.

In conclusion, using CGEA of esophagectomy specimens, we identified 75 genes that are characteristically expressed in BSCE; a gene expression scoring system constructed from these data enabled us to distinguish BSCE from non-BSCE with high sensitivity and specificity, even on endoscopic biopsy specimens. We believe that this scoring system can be a novel method that may significantly contribute to improving the diagnostic accuracy for BSCE.

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