

transferred to Immobilon-P Transfer membranes (0.45 μm) (Millipore, Bedford, MA) in a tank-transfer apparatus, and membranes were blocked with Immuno Block (DS Pharma Biomedical Co. Ltd.). Anti-GP2 (SIGMA-Aldrich, Japan) diluted 1:200 and anti-MFAP4 (Proteintech Group, Inc.) diluted 1:1000 in blocking buffer, which were used as primary antibodies. Goat antirabbit IgG horseradish peroxidase (Invitrogen Carlsbad, CA) diluted 1:3000 in blocking buffer was used as a secondary antibody. Antigens on membranes were detected with enhanced chemiluminescence detection reagents (GE Healthcare).

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections were obtained from the Osaka Medical Center for Cancer & Cardiovascular Diseases. Sections were deparaffinized and rehydrated followed by antigen unmasking in 10 mM citrate buffer (pH 6.0) for 10 min at 15 psi and 120 °C followed by treatment with 0.3% H_2O_2 (Wako). After washing three times with PBS, nonspecific binding of antibodies was blocked with blocking buffer (3% BSA/PBS) for 1 h. Tissues were then incubated for 1 h with anti-GP2 diluted 1:750 in 3% BSA/PBS. After washing with PBS, DAB (DAKO Japan, Kyoto, Japan) was used to visualize tissue antigens according to the manufacturer's instructions. Tissue sections were counterstained with Mayer's Hematoxylin Solution (WAKO Pure Chemical Industries Ltd.) for 30 s and dehydrated with 100% ethanol and xylene (WAKO), and coverslips were mounted with Malinol (Muto Pure Chemicals, Tokyo, Japan). All slides were examined and scored by two of the authors who were blinded to clinical data of patients. Staining intensity was recorded on the following scale: 0, no staining was observed, or cytoplasm staining was observed in less than 10% of tumor cells; 1, faint/barely perceptible cytoplasm staining was detected in more than 10% of tumor cells (cells exhibited incomplete cytoplasm staining); 2, weak or moderate cytoplasm staining was observed in more than 10% of tumor cells or strong cytoplasm staining in less than 30%; and 3, strong cytoplasm staining was observed in more than 30% of tumor cells.

RESULTS

MS-based technologies have been applied to many large-scale proteomics studies and mostly applied to the discovery of protein biomarkers, especially in the field of cancer. These results have produced numerous candidate protein biomarkers. Unfortunately, only a few are currently being validated and applied in clinical prognostics. Consequently, we tried to perform proteomics analysis using a discovery-through-verification strategy. An overview of the method for verification of prognostic biomarkers and its application to the model of breast cancer prognostic biomarkers is shown in Figure 1. In the discovery phase, we analyzed pooled membrane protein fractions isolated from high ($n = 9$) or low-risk ($n = 9$) breast cancer tissues by LC-MS/MS using iTRAQ, to generate data of candidate protein biomarkers. We identified a total of 5122 unique proteins. A list of proteins is presented in Supplementary Table 2 (Supporting Information). The identified 5122 proteins were examined with respect to cellular localization using Gene Ontology annotation analysis in Ingenuity pathway analysis (IPA) or The Database for Annotation, Visualization and Integrated Discovery (DAVID) (Figure 2A). IPA or DAVID revealed that 2480 (48.4%) were annotated to membrane proteins, 826 (16.1%) proteins were plasma membrane, and 340 (6.6%) proteins were extracellular space proteins by GO analysis. Furthermore, of the identified proteins, 1469 (28.7%) proteins were predicted to have transmembrane

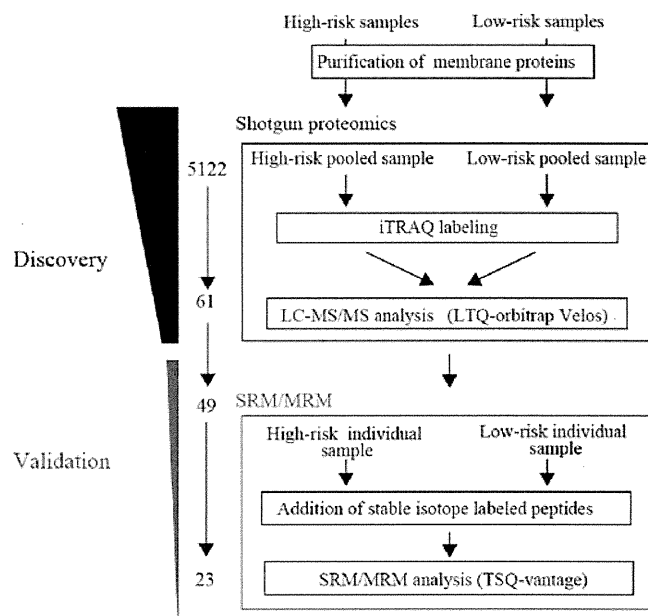


Figure 1. Overview of the workflow used to discovery and verify candidate biomarkers, showing the flux of candidates at each stage of the pipeline. For the discovery step, an iTRAQ discovery study in which peptides, and thereby proteins that are differentially expressed among pools of high-risk patients ($n = 9$) and low-risk patients ($n = 9$), are identified. For the verification step, a targeted SRM/MRM study was set up based on the information found in the prior study to validate quantitative findings from the iTRAQ discovery study.

(A)

Location	Numbers	%
total	5122	
Membrane	2480	48.4
Plasma membrane	829	16.2
Extracellular Space	340	6.6

(B)

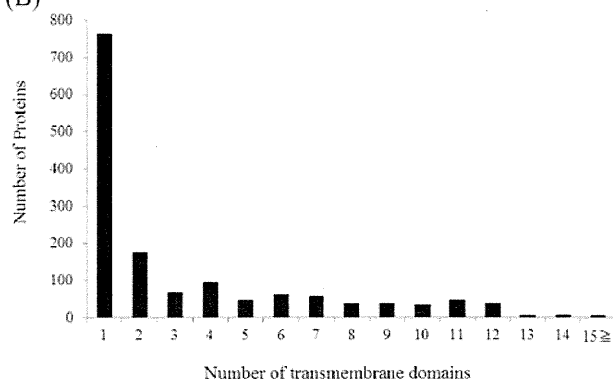


Figure 2. (A) Sub cellular classification of proteins identified based on annotation with gene ontology. (B) Numbers of proteins with transmembrane helices predicted by the TMHMM2.0 algorithm.

segments via TMHMM2.0 algorithm. A histogram of the generated data is presented in Figure 2B. These results support the effectiveness of the method to solubilize and digest integral membrane proteins containing transmembrane domains, allowing large-scale detection and identification of this protein class with no bias against membrane proteins. Fold changes were

Table 1. List of Proteins for which SRM/MRM Assays Were Developed^{a,b}

high-risk > low-risk ^c				high-risk < low-risk ^d			
accession no	protein name	gene name	ratio (low-risk/high-risk)	accession no	protein name	gene name	ratio (low-risk/high-risk)
P26022	pentraxin 3, long	PTX3	0.166	Q9UHI5	solute carrier family 7 (amino acid transporter, L-type), member 8	SLC7A8	2.091
O75762	transient receptor potential cation channel, subfamily A, member 1	TRPA1	0.215	P23142	fibulin 1	FBLN1	2.119
Q5H943	chromosome X open reading frame 61	KKLC1	0.264	Q05707	collagen, type XIV, alpha 1	COL14A1	2.12
Q9NYZ1	family with sequence similarity 18, member B1	FAM18B2	0.269	Q9P0K1	ADAM metalloproteinase domain 22	ADAM22	2.148
Q9H313	tweety homologue 1	TTYH1	0.322	Q14956	glycoprotein (transmembrane) nmb	GNPMB	2.154
Q9NQG1	mannosidase, beta A, lysosomal-like	MANBAL	0.327	Q99542	matrix metalloproteinase 19	MMP19	2.172
P02458	Collagen, type II, alpha 1	COL2A1	0.354	P38185	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 6	SERPINA6	2.18
P50443	solute carrier family 26 (Sulfate transporter), member 2	SLC26A2	0.36	Q9BXN1	asporin	ASPN	2.222
P26012	integrin, beta 8	ITGB8	0.367	O60938	keratocan	KERA	2.223
P15328	folate receptor 1 (adult)	FOLR1	0.381	Q15661	tryptase alpha/beta 1	TPSAB1	2.23
O43490	prominin 1	PROM1	0.409	O43927	chemokine (C-X-C motif) ligand 13	CXCL13	2.269
Q01650	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	SLC7A5	0.454	O99983	osteomodulin	OMD	2.405
Q9HBA0	transient receptor potential cation channel subfamily V, member 4	TRPV4	0.462	O43692	peptidase inhibitor 15	PI15	2.42
P53985	solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	SLC16A1	0.47	O76061	stanniocalcin 2	STC2	2.51
Q9NP58	ATP-binding cassette subfamily B member 6, mitochondrial	ABCB6	0.488	P25189	myelin protein zero	MPZ	2.53
				P20774	osteoglycin	OGN	2.543
				Q53GD3	solute carrier family 44, member 4	SLC44A4	2.623
				P27658	collagen, type VIII, alpha 1	COL8A1	2.93
				P29120	proprotein convertase subtilisin/kexin type 1	PCSK1	2.951
				P01009	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	SERPINA1	3.037
				P02743	amyloid P component serum	APCS	3.324
				P55083	microfibrillar-associated protein 4	MFAP4	3.392
				P25311	alpha-2-glycoprotein 1, zinc-binding	AZGP1	3.773
				P55259	glycoprotein 2(zymogen granule membrane)	GP2	3.864
				P05060	chromogranin B (secretogranin 1)	CHGB	4.008
				P15086	carboxypeptidase B1(tissue)	CPB1	4.306
				P05090	apolipoprotein D	APOD	4.887
				P12273	prolactin-induced protein	PIP	6.033

^aThe overall protein ratios identified by iTRAQ are indicated for each protein. ^bThese values are based on total peptide information obtained for each protein. ^cProtein whose expression in the high-risk group is 2-fold or more than that in the low-risk group. ^dProtein whose expression in the low-risk group is 2-fold or more than that in the high-risk group.

determined based on the ratio of peak areas of iTRAQ reporter ions for the corresponding peptides from high and low-risk samples. Among 2480 membrane proteins, 188 showed >2-fold differences between high and low risk groups (either up-regulated or down-regulated), of which 61 proteins were annotated to plasma membrane and extracellular proteins.

The next step, to perform verification of 61 candidate protein biomarkers in individual patient tissue samples (high-risk; $n = 9$, low-risk; $n = 7$), we carried out relative quantification by SRM/MRM using SI-peptides as internal standards (Figure 1). In a typical SRM/MRM assay, a unique peptide is measured, and its concentration is assumed to be equal to the concentration of its parent protein. In this work, proteotypic peptides were chosen based on shotgun proteomic identification data. Peptides that had modifications, such as partially oxidized methionine, were avoided and when possible, two peptides were used per protein. For SRM/MRM, 49 of 61 differentially expressed proteins were selected by the above criteria. Finally, 86 peptides representing 49 proteins (21 plasma membrane and 28 extracellular proteins) remained in the list (Table 1). To obtain data for making transitions for each peptide in SRM/MRM, we analyzed each

SI-peptide using LTQ-Orbitrap XL. Four transitions, γ -ions, were chosen for each peptide. A total of 688 transitions were used for targeting 86 peptides of 49 proteins. The complete transition list can be found in Supplementary Table 3 (Supporting Information). Individual membrane fraction samples were digested and analyzed in duplicate. The SRM analysis successfully verified all 49 target proteins. Among them, 23 proteins were differentially expressed between high-risk and low-risk groups; 21 were increased and 2 were decreased in low risk group. Of these 15 of the target analytes showed a significant difference (ratio > 2, $p < 0.05$) between two groups (Figure 3, Supplementary Table 4A, Supporting Information) and 8 showed apparent, but not significant, difference between high and low risk breast cancer tissues (Supplementary Table 4B, Supplementary Figure 2, Supporting Information). On other hands, the 26 proteins showed no significant difference between high and low-risk breast cancer tissues (Supplementary Table 5, Supporting Information). Proteins with significant difference mentioned above included the following 10 proteins that have been reported with altered expression in breast cancer by other methods: SERPINA3, APOD, APCS, SERPINA1, LUM,

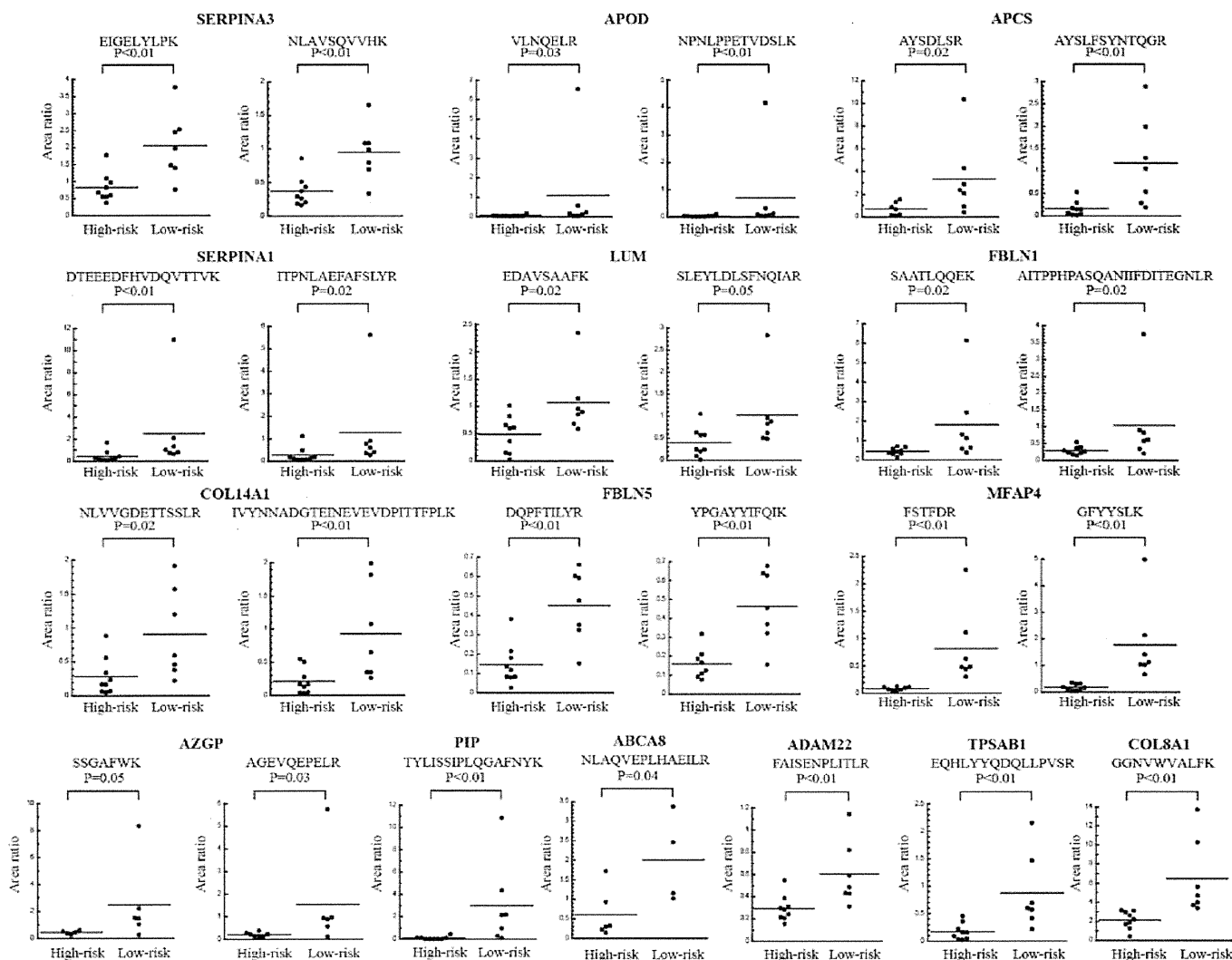


Figure 3. Quantitation of targeted peptide expression in the membrane fraction which showed a significant difference between high-risk and low-risk patients. Area ratio (endogenous peptide/SI-peptide) of each peptide in individual patient tissue samples (high-risk; $n = 9$, low-risk; $n = 7$) were subjected to nonparametric analysis of the Wilcoxon test with a cut off of $p < 0.05$.

FBLN1, FBLN5, AZGP1, PIP, and COL8A1.^{26–35} However, the following 5 proteins have not been reported in breast cancer: COL14A1, MFAP4, ABCA8, ADAM22, and TPSAB1. These proteins may be novel prognostic biomarker candidates in breast cancer.

To confirm the differential expression observed by SRM/MRM analysis, the expression level of a selected protein was further examined using Western blotting. We selected candidate proteins on the basis of extent of significant differential expression and antibody availability. MFAP4 was thus selected and Western blotting was performed with membrane fractions of individual patient tissue samples. Western blot results are shown in Figure 4B. Results showed a positive correlation with SRM/MRM analysis results. Thus, SRM/MRM analysis is expected to be an effective method for verification from numerous candidate protein biomarkers.

Among eight proteins with apparent, but not significant, difference between high and low risk breast cancer tissues (Supplementary Table 4B, Supplementary Figure 2, Supporting Information), known markers of breast cancer such as STC2 and ITGB8 has been shown to be differently expressed.^{36–38} However, to our knowledge, the other 6 proteins have not been

reported in breast cancer: KERA, OMD, SERPINA6, PTX3, PI15, and GP2. Among them, we examined the expression level of the GP2 protein by Western blotting and IHC because a good antibody is available. The expression of GP2 was significantly higher in the low-risk group than that of the high-risk group by either Western blotting or IHC (Figure 5). The above results indicated that GP2 and MFAP4 are potential prognostic biomarkers for breast cancer.

DISCUSSION

The aim of this study was to establish a discovery-through-verification pipeline of large scale proteomics analysis using limited tissue samples. In this study, we identified and verified several biomarker candidates predicting recurrence risk of breast cancer by tissue membrane proteomic analysis using an established strategy. In total, 5122 proteins were identified with high confidence by a quantitative proteomics approach using iTRAQ labeling, 2480 protein (48.4%) of them were annotated as membrane proteins, 829 proteins (16.1%) were plasma membrane and 340 proteins (6.6%) were extracellular space proteins by GO analysis. Also 1469 proteins (28.7%) were predicted to have transmembrane domain by TMHMM algorithm.

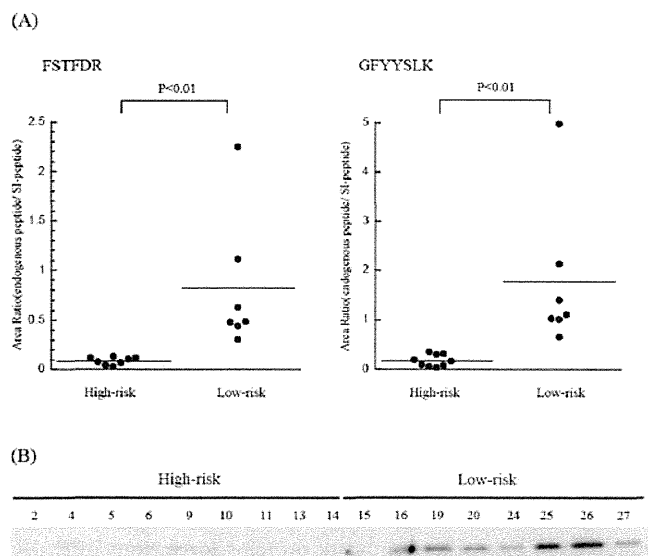


Figure 4. SRM/MRM and Western blot analysis data confirm elevations of MFAP4 in low-risk breast cancer patients. (A) Distributions of area ratio (endogenous peptide/SI-peptide) of MFAP4 in human breast cancer with high-risk and low-risk. The median value is plotted as a line. Results derived from SRM-MS based measurements. (B) Western blot analysis showing higher levels of MFAP4 in low-risk patients than those of high-risk patients. The numbers indicate the patient numbers in Supplementary Table 1, Supporting Information.

A total of 61 proteins were found to be altered by 2-fold or more between high and low-risk breast cancer tissues and 49 of these

proteins were subsequently verified with targeted proteomics using SRM/MRM. Twenty-three proteins were shown to be differentially expressed between the two groups. Additionally, two of these proteins, MFAP4 and GP2, were further validated to be differentially expressed between the different groups using Western blotting and IHC.

We showed that the combination of iTRAQ shotgun and SRM/MRM proteomics is a powerful technique for identification and verification of numerous biomarker candidates. At the discovery stage of this study, human patient tissue samples were pooled within each group to perform effective screening of differentially expressed proteins across breast cancers of each group using limited amounts of specimen. Additionally, for verification from numerous candidate protein biomarkers, we performed SRM/MRM analysis using individual patient tissue samples. This combination approach was able to discover and verify a novel candidate biomarker with high-throughput. Thingholm and co-workers published a large-scale quantitative proteomic analysis using iTRAQ combined with SRM for discovery and validation of biomarkers for type 2 diabetes.⁸ However, their targeted proteomics were performed without SI-peptides. The difficulty with SRM/MRM analysis may occur without internal standards, which provide reference signals for the verification of analyte specificity.⁷ The use of SI-peptide provides the most favorable SRM/MRM information for each peptide, such as highest intensity fragment ions and peptide elution time. The target peptide area ratio is then determined by measuring the observed area value for the target peptide relative to that of the SI-peptide. Therefore, we used crude SI-peptides that can be synthesized at a lower cost than purified synthesized peptides. As a consequence, our methods to rapidly

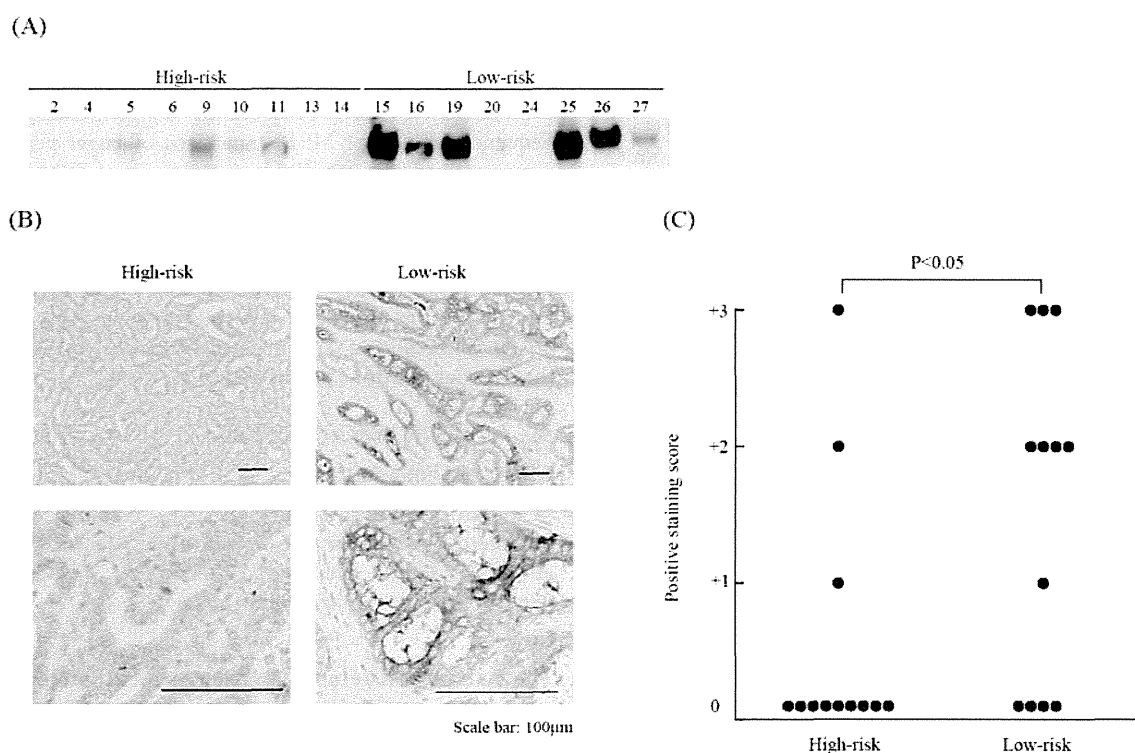


Figure 5. Western blot and IHC analysis data confirm elevations of GP2 in low-risk breast cancer patients. (A) Western blot analysis showing higher levels of GP2 in low-risk patients than those in high-risk patients. The numbers indicate the patient numbers in Supplementary Table 1 (Supporting Information). (B) Immunohistochemistry staining of GP2 on high-risk and low-risk breast cancer specimens. Top is $\times 200$ and bottom is $\times 400$. (C) Staining intensity of 24 samples (12 high-risk groups and 12 low-risk groups) was examined and scored according to Materials and Methods. Statistical analysis was performed using the Wilcoxon–Mann–Whitney test with a cut off of $p < 0.05$ in (B).

generate a SRM/MRM assay using crude synthetic peptides expands the application of SRM based targeted MS for high-throughput protein detection and quantification.³⁹

Several previously published reports have described membrane proteome analysis.^{13,40,41} In a recent study, Polisetty and co-workers carried out a large-scale discovery study in which they utilized a technology combining iTRAQ and LC-MS/MS and identified 1834 distinct proteins from membrane fractions of glioblastoma multiforme patient specimens, 56% of them (1027) are annotated as membrane protein.¹² In this study, we identified a total of 5122 proteins in the membrane fraction, 48% of them (2480) are known membrane proteins associated with major cellular processes and this number of membrane proteins was much greater than those previously reported. This was because we performed a method that utilized a combination of PTS method-based isolation of membrane proteins and iTRAQ method. In the membrane preparation stage, efficient isolation of membrane proteins was achieved using ultra centrifugation and subsequent PTS. In the cleavage procedure of membrane proteins, PTS method allowed the use of a high detergent concentration to achieve efficient solubilization of very hydrophobic membrane proteins while avoiding interference with subsequent iTRAQ-LC-MS/MS analysis.⁴² In this SRM/MRM assay, we found that a number of proteins were detected in the membrane fraction, but not in the total fraction (data not shown). Thus, this method may provide deeper proteome coverage for identification of tissue membrane proteins.

In our study, eight proteins were not detected in several patient samples with this SRM/MRM analysis (Supplementary Table 4B, Supplementary Figure 2, Supporting Information). Consequently, they could not be subjected to the Wilcoxon test. However, GP2 showed a significant difference between high-risk and low-risk tissues in breast cancer using Western blotting and IHC (Figure 5). In SRM/MRM, there are several possible reasons why not all targeted proteins were detected. They include 1) the absence of targeted protein in samples, that is no expression in the patient tissues used, (2) the targeted protein was present but its expression level was below the limit of quantitation, and (3) the MS peak of the target protein was hard to detect due to high background noise. In this study, expression of undetected proteins may be below the limit of quantitation. Thus, although the expression of GP2 protein could not be verified by SRM/MRM, Western blotting and IHC results showed a significant difference between high-risk and low-risk tissues in breast cancer.

A number of commercialized multigene prognostic and predictive tests have entered the complex and expanding landscape of breast cancer prognostics. The two assays that have achieved the most practical success are oncotype DX and MammaPrint. However, it has a high cost. In contrast, commercialized IHC multigene predictors are less expensive.⁴³ Cheang et al. reported that the immunohistochemical determination of ER, PgR, HER2, and Ki67 indexes are able to distinguish the luminal B subgroup with poor prognosis from the luminal A subgroup with good prognosis.²⁵ This assay was recommended at St-Gallen in 2011.⁴⁴ To our knowledge, most prognostic and predictive biomarkers show stronger staining in the high-risk group than that of the low-risk group. However, we found GP2 and MFAP4 were highly stained in the low-risk group, which could define this group. Consequently, combining this biomarker with existing prognostic tools (ER/PgR, Ki67, HER2) should be able to predict prognosis of breast cancer more accurately.

MFAP4 and GP2 show a significant differential expression between high and low-risk groups in breast cancer. MFAP4 was initially identified as a gene commonly deleted in the contiguous gene syndrome Smith-Magenis.⁴⁵ MFAP4 was involved in calcium-dependent cell adhesion or interactions with Integrin, Pulmonary surfactant protein A, or Lung surfactant protein D.^{46–48} To our knowledge, there has been no report to show the expression and function of MFAP4 on cancer. GP2 is the major membrane protein present in pancreatic zymogen granules, and is cleaved and released into the pancreatic duct along with exocrine secretions.^{49–51} Recently, Hase et al. reported that GP2, specifically expressed on the apical plasma membrane of M cells among enterocytes, serves as a transcytotic receptor for mucosal antigen.⁵² However, there has been no report about GP2 protein expression on cancer tissues until now. Further investigation will be necessary to clarify the function of MFAP4 and GP2 on cancer.

In conclusion, although numerous studies have identified hundreds of biomarker candidate proteins for various diseases, few of them have performed large scale verification due to the unavailability of antibodies with high quality. This study showed that the iTRAQ and SRM based discovery-through-verification strategy contributes to generate clinically useful biomarkers for various diseases, including cancer. It may be possible to replace current clinical examinations by this latest proteomic technology in the near future.

■ ASSOCIATED CONTENT

📄 Supporting Information

Supplementary Figure 1. A peak profile for SRM transitions of MFAP4: GFYYSLK. Supplementary Figure 2. Quantitation of eight proteins expression which showed apparent, but not significant, difference between high and low risk breast cancer tissues. Supplementary Table 1. Clinical features of breast cancer patients. Supplementary Table 2. A list of identified and quantified proteins by iTRAQ proteomics. Supplementary Table 3. Sequences and SRM/MRM transitions of target peptide for 49 proteins. Supplementary Table 4. Summary of SRM/MRM data; (A) proteins with significant different expression between high and low risk breast cancer tissues; (B) proteins with apparent, but not significant, difference between high and low risk breast cancer tissues. Supplementary Table 5. Summary of SRM/MRM data; proteins with no significant difference between high and low risk breast cancer tissues. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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Sports, and Culture of Japan. We thank Dr. Yasuko Nishizawa for advice with immunohistochemistry.

■ ABBREVIATIONS

iTRAQ, isobaric peptide tags for relative and absolute quantification; SRM, selected reaction monitoring; MRM, multiple reaction monitoring; PTS, phase-transfer surfactants; SI-peptide, stable isotope-labeled peptide; CID, collision-induced dissociation; HCD, higher energy collision-induced dissociation; IHC, Immunohistochemistry; LC-MS/MS, liquid chromatography tandem mass spectrometry; CE, Collision energy; LTQ, linear ion trap; fwhm, full wide at half maximum; FDR, false discovery rate.

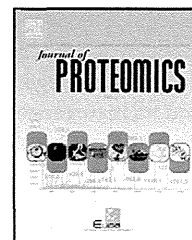
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Plectin promotes migration and invasion of cancer cells and is a novel prognostic marker for head and neck squamous cell carcinoma

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ABSTRACT

Head and neck squamous cell carcinoma (HNSCC) is usually found at a late stage and distant metastasis occurs at high frequency; therefore, novel prognostic markers are needed. This study was aimed to identify novel tumor markers in HNSCC. We identified 65 proteins which were significantly increased or decreased in the tumors by 2D-DIGE using 12 HNSCC and adjacent non-cancer tissues. Western blotting and immunohistochemical analysis confirmed that the expression of plectin was significantly increased in most cancer tissues as compared with non-cancer tissues. Strikingly, the suppression of endogenous plectin using siRNA inhibited the proliferation, migration and invasion of HNSCC cells and down-regulated Erk 1/2 kinase. Furthermore, immunohistochemistry using paraffin-embedded tissues from 62 patients showed not only that the frequency of recurrence was correlated with the plectin expression but that the prognosis of patients with a high plectin was extremely poor. Moreover, the survival rate of patients with a high plectin was significantly lower than that of patients with low E-cadherin levels, which is known to correlate with the poor prognosis of HNSCC. Our findings suggest that plectin promotes the migration and invasion of HNSCC cells through activation of Erk 1/2 kinase and is a potential prognostic biomarker of HNSCC.

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1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world, with a 5-year overall

survival rate of approximately 40–50% [1,2]. Despite the improvement of therapies involving surgery, radiotherapy and chemotherapy, the prognosis of HNSCC patients in advanced stages is poor for local tumor recurrence and distant

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metastasis [3]. Most prognosis factors fail to provide definitive information regarding the biological behavior of the tumor and its recurrence and metastasis potential. Identifying a biomarker that correlates with recurrence and metastasis would provide more accurate information on prognosis and enable a more aggressive therapy to be selected for high-risk patients.

Proteomic technologies have been used to identify cancer-specific proteins that are useful for cancer diagnosis, prognosis, and therapeutic targets in HNSCC [4]. Although extensive proteome analysis has identified numerous proteins overexpressed in various cancer tissues, very few markers have become available for routine clinical use, mainly because potential candidates have not been detected due to their low abundance and/or validated extensively by other methods [5]. Two-dimensional difference gel electrophoresis methods (2D-DIGE) have been developed to overcome this problem [6–8]. We have previously identified several novel proteins with altered expression in colorectal, esophageal and liver cancers using this method [6–9].

In this study, we aimed to identify novel biomarkers to predict the clinical outcome of HNSCC patients. Using agarose 2D-DIGE, we compared protein expressions between head and neck cancer tissues and non-cancer tissues. Among several proteins identified to be differentially expressed between cancer and non-cancer tissues, we found that plectin is involved in the migration and invasion of HNSCC cells and is a novel prognostic marker for HNSCC.

2. Materials and methods

2.1. Human tissue samples and cell lines

Tissues from 12 patients with primary HNSCC were resected surgically without any neo-adjuvant therapy in the Department of Otorhinolaryngology, Head and Neck Surgery, Chiba University Hospital (Table 1). The ethics committee of the Graduate School of Medicine, Chiba University approved the protocol. Written informed consent was obtained from each patient before surgery. Excised samples were obtained within 60 min after the operation and were immediately placed in liquid nitrogen and stored at -80°C . A human

HNSCC-derived cell line D562 was obtained from the Human Science Research Resources Bank (Osaka, Japan). Cell lines were grown in IMDM with 10% FBS.

2.2. Protein extraction and proteomic analysis

Protein extraction, agarose 2D-DIGE and enzymatic in-gel digestion of proteins were performed as described previously [7,8]. To identify proteins, digested peptides were injected into a trap column: 0.3×5 mm L-trap column (Chemicals Evaluation and Research Institute, Saitama, Japan), and an analytical column: 0.1×50 mm monolith column (AMR, Tokyo, Japan), which was attached to the Chorus 220 HPLC system (AMR). Purified peptides were introduced from HPLC to Q-star pulser i (Applied Biosystems, Foster City, CA, USA) at 500 nL/min. The MASCOT search engine (version 2.0.5, Matrixscience, London, UK) was used to identify proteins from the mass and tandem mass spectra of peptides. Peptide mass data were matched by searching the National Center for Biotechnology Information Human database (NCBI nr 20080210, February 2008, 199851 entries). Database search parameters were: the charge (z) of the precursor ion, 2+ and 3+; peptide mass tolerance, 1.2 Da; fragment tolerance, 0.5 Da; enzyme was set to trypsin, allowing up to one missed cleavage; variable modifications, methionine oxidation. The minimum criterion of the probability-based MASCOT/MOWSE score was set with 5% as the significant threshold level. We considered >1.5 in spot volume to be significant according to previous reports as follows. Data set of all spots volumes obtained from six Cy3/Cy5 same sample showed robust thresholds of -2.33 and $+1.52$ for 90% confidence [10]. Furthermore, based on the observation that 2 S.D. ranged from 1.31 to 1.52, gel features changing by >1.5 in spot volume were considered significant [11].

2.3. Western blotting and immunohistochemistry

Western blotting (WB) and immunohistochemistry (IHC) were performed as described previously [7,8]. Anti-plectin goat polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:500 for WB and 1:50 for IHC, anti-periplakin rabbit polyclonal antibody (Bethyl Laboratories, Montgomery, TX) diluted 1:2000 for WB and 1:200 for IHC, anti-envoplakin mouse monoclonal antibody (Santa Cruz Biotechnology Inc.) diluted 1:1000 for WB and 1:100 for IHC, anti-comulin (CRNN) rabbit polyclonal antibody (Proteintech Group, Chicago, IL) diluted 1:3000 for WB and 1:300 for IHC, and anti- β -actin goat polyclonal antibody (Santa Cruz Biotechnology Inc.) diluted 1:500 in blocking buffer were used as primary antibodies. Anti-integrin $\beta 4$ antibody (Santa Cruz Biotechnology Inc.) diluted 1:50, and anti-E-cadherin antibody (Santa Cruz Biotechnology Inc.) diluted 1:50 were used for IHC.

All slides were examined by two of the authors (KK and TT) who were blinded to the clinical data. When the results were discordant, the judgment was made by the other investigator. Staining intensity was recorded on the following scale: 0, no staining is observed, or cytoplasm staining is observed in less than 10% of the tumor cells; 1+, faint/barely perceptible cytoplasm staining is detected in more than 10% of tumor

Table 1 – Clinical features of patients with HNSCC.

Sample number	Age	Gender	Location	UICC TN	UICC stage
1	66	Female	Hypopharynx	T4aN0	IVA
2	72	Male	Hypopharynx	T2N3	IVB
3	71	Male	Hypopharynx	T1N0	I
4	60	Male	Hypopharynx	T3N2c	IVA
5	69	Male	Hypopharynx	T3N2c	IVA
6	62	Male	Hypopharynx	T2N1	III
7	52	Female	Hypopharynx	T4aN2b	IVA
8	60	Male	Hypopharynx	T2N2c	IVA
9	81	Female	Hypopharynx	T4aN2b	IVA
10	74	Female	Hypopharynx	T4aN2b	IVA
11	66	Male	Hypopharynx	T4aN2b	IVA
12	64	Male	Hypopharynx	T4aN2b	IVA

cells (the cells exhibit incomplete cytoplasm staining); 2+, weak or moderate cytoplasm staining is observed in more than 10% of tumor cells or strong cytoplasm staining in less than 30%; and 3+, strong cytoplasm staining is observed in more than 30% of tumor cells (Fig. S1). For statistical analysis, these categories were pooled (0 and 1+, “low”; 2+ and 3+, “high”). Positive and negative control slides were included in all experiments.

2.4. Gene knockdown using siRNA

The target sequences for plectin RNA interference were as follows: plectin siRNA1: 5'-CCA AGA ACT TGC AGA AGT T-3', plectin siRNA2: 5'-CTG AGA ACC GCG CAC TCA T-3' purchased from Sigma Aldrich Japan (Tokyo, Japan).

2.5. Cell proliferation assay

D562 HNSCC cells in 24-well plates were transfected with siRNA (20nM final concentration). Both attached and floating cells were corrected with trypsinization. After staining with Trypan blue, the number of Trypan blue-positive cells was counted on days 2, 4, 6 after transfection.

2.6. Wound healing assay

Cell migration ability was evaluated by a wound healing assay. D562 cells were plated in 12-well dishes at a density of 2×10^6 cells and transfected with siRNA (20nM final concentration). After overnight incubation, an artificial wound was carefully created at 0 h using a 200 μ l pipette tip to scratch the confluent cell monolayer. A photomicrograph was taken immediately after scratching (time 0 h) and 6 h and 24 h later.

2.7. Matrigel invasion assay

The invasion assay was carried out using the BioCoat Matrigel Invasion Chamber kit (Becton Dickinson Bioscience, Bedford, MA) according to the manufacturer's instructions. D562 cells were harvested, resuspended in FBS-free IMDM and seeded in a Matrigel invasion chamber or a control insert (2×10^5 cells/well). Lower chambers were filled with culture medium containing 10% FBS as a chemoattractant. After dispersed cells were cultured at 37 °C for 22 h, cells on the upper side of the membrane were then removed using a cotton swab and the filters were washed, fixed and stained using the Diff-Quick kit (Sysmex, Kobe, Japan). Percent invasion was represented as mean number of cells invading through a Matrigel insert membrane relative to that passing through a control insert membrane.

2.8. Statistical analysis

All data were analyzed using the statistical software SPSS11.0 for Windows. Overall survival was analyzed by the Kaplan-Meier method and the results were compared by log-rank test. Univariate analysis was performed for the correlation between overall survival time and various clinical characteristics, including gender, age, WHO histological type, clinical stage, and the expression levels of plectin, integrin $\beta 4$ and

E-cadherin. The significance of variables for survival was analyzed by the Cox proportional hazards model in multivariate analysis. The χ^2 test and Student's t test were used to compare clinicopathologic data. Statistical significance was assumed when <0.05 .

3. Results

3.1. Identification of differentially expressed proteins in human HNSCC tissue

To identify novel markers useful for the diagnosis or prognosis of HNSCC, we used the agarose 2D-DIGE method to compare the profiling of protein expression between adjacent normal epithelial tissues (Cy3) and HNSCC tissues (Cy5) with

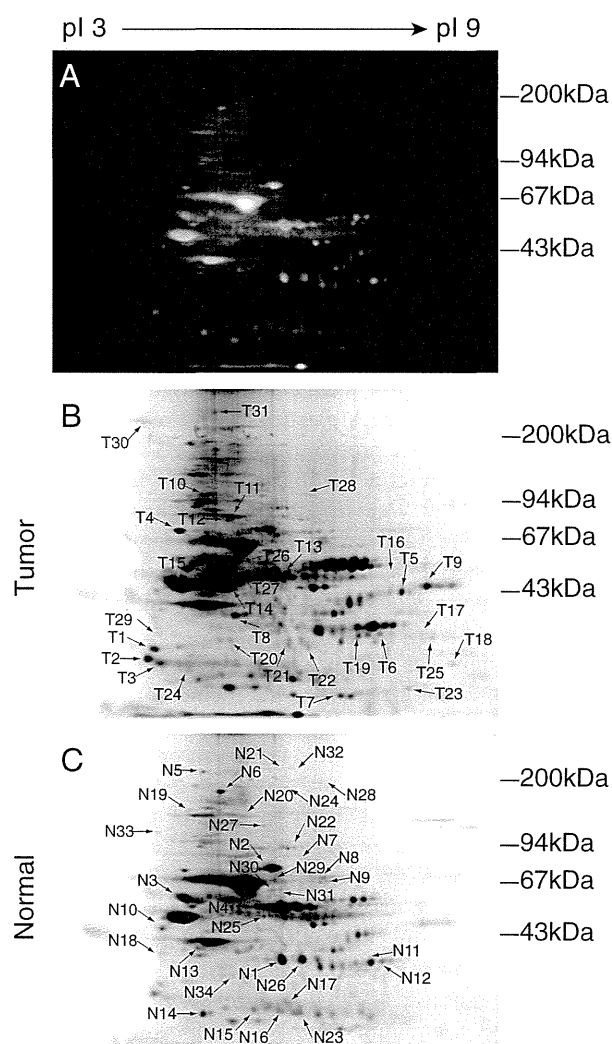


Fig. 1 – Proteomic analysis of HNSCC tissues using agarose 2-D DIGE. A, Increased protein spots in cancer tissues are displayed in red (Cy5), and decreased protein spots in cancer tissues in green (Cy3). B and C, conventional agarose 2-DE patterns of HNSCC (B) and non-cancer (C) were visualized by CBB staining.

a mixed-sample internal standard (Cy2) (Fig. 1A). Protein spots that were increased or decreased in tumor tissues were displayed as red or green, respectively. These spots were detected and quantitated with DeCyder imaging analysis software, and then statistical analysis was performed across the 12 gels. All of the samples were examined in duplicate or triplicate, and about 2500 protein spots (minimum 2297 and maximum 2518) were detected consistently in each gel. We considered >1.5 in spot volume to be significant as described in Material and Methods. The fluorescence volumes of 39 spots increased and 60 spots decreased in cancer tissues compared with adjacent normal epithelial tissues (Student's *t* test, $p < 0.05$). To identify proteins, 300 μg protein in whole-cell lysate was separated by conventional agarose 2-DE and proteins were visualized by Coomassie blue staining (Fig. 1B, C). Of 99 spots, 88 (65 proteins) were identified by mass spectrometry (Table 2, 3, Fig. S2). Heat shock 27 kDa protein 1 was identified in both up-regulated (T27) and down-regulated proteins (N15) in HNSCC with a distinct pI value, which suggests that the protein was differently modified, probably phosphorylated, in cancer and normal tissues.

3.2. Validation of the identified proteins by Western blotting and immunohistochemistry

Among the 65 proteins with altered expression, we mainly focused on proteins, whose expression levels have not been well studied in head and neck cancers. The expression level of the four proteins (periplakin, envoplakin, cornulin, and plectin) with commercially available antibodies was examined by Western blotting. As shown in Fig. 2A, periplakin, envoplakin, and cornulin were significantly down-regulated in HNSCC tissues, whereas plectin was up-regulated, which confirms the results of proteomic analysis. The relative expression levels of plectin normalized with β -actin level are indicated below the Western blotting image. Although the same amount of protein was loaded in each lane, the expression level of β -actin was different in some of the cancer and normal tissue pairs, which might reflect the different expression of β -actin in these tissues.

Although there was no bias in the cellularity of normal and tumor tissues, whole tissue sections included non-epithelial components, and the altered protein expression in our 2D-DIGE analysis may have resulted from non-epithelial components. Thus, differential protein expression in HNSCC was validated by immunohistochemistry to examine the localization of identified proteins. Immunohistochemical validation studies were performed on paraffin-embedded samples from patients with cancer tissues and non-cancer tissues using four antibodies (plectin, periplakin, envoplakin, and cornulin). Although no staining of plectin was observed in normal squamous cells, tumor cells showed uniform staining in the cytoplasm and cell membrane (Fig. 2B). Periplakin, envoplakin and cornulin all showed strong and uniform staining in the cytoplasm of normal squamous cells; in particular, the horny layer and granular layer were strongly stained by anti-cornulin antibody. In marked contrast, faint, weak staining of proteins was observed in tumor cells (Fig. 2B). Examination of several tissue sections showed similar staining patterns, which indicates that altered expression of these proteins

reflects actual changes in the protein level in cancer cells and not in stromal cells.

3.3. Plectin knockdown inhibits proliferation, migration, and invasion of HNSCC cell lines

Using proteomic analysis, we confirmed that several proteins were differentially expressed in HNSCC tissues. Among them, only plectin was upregulated, which could be a suitable biomarker for the diagnosis of HNSCC. Thus, we concentrated on plectin and investigated the functional role of plectin in HNSCC development.

First, we examined if plectin contributes to cell proliferation in HNSCC using the RNAi technique. After introduction of plectin siRNA into D562 cells for 48 hr, suppression of plectin was confirmed by Western blotting (Fig. 3A), while GL2 siRNA had no substantial effect on endogenous plectin expression. Then, the proliferation of D562 cells was examined by cell counting 2–6 days after siRNA treatment. As a result, cell proliferation was significantly reduced by plectin knockdown (Fig. 3B).

Penetration of the extracellular matrix and basement membrane by cancer cells is a key step in tumor invasion. Moreover, cell migration is essential for the invasion of cancer cells. Since plectin is a cross-linking protein of intermediate filaments, microtubules and actin microfilaments, it could be involved in tumor cell migration and invasion. In fact, plectin interacts with integrin $\beta 4$, which has been reported to correlate with migration and invasion in HNSCC [12–14]. We therefore examined if plectin contributes to such cellular processes.

The effect of plectin knockdown on the migration potency of HNSCC cells was determined using the scratch-wound healing assay. The extent of wound closure can be taken as a direct measure of cell motility or migration capacity. Closure of the wound was almost complete in D562 cells carrying control siRNA within 24 hr of transfection, but the wound still existed in D562 cells into which plectin siRNA had been introduced. Thus, cell motility was significantly suppressed by plectin knockdown (Fig. 3C,D).

Next, the effect of plectin knockdown on HNSCC cell invasion was examined using the Matrigel invasion assay. Mock cells and plectin knockdown cells passed similarly through a control insert (Fig. 3E, upper), but the number of cells passing through the Matrigel insert membrane was markedly reduced in plectin knockdown invasive cells as compared with mock invasive cells (Fig. 3E, lower). Percent invasion of plectin siRNA-treated cells was significantly lower than that of mock or control siRNA-treated cells (Fig. 3F); therefore, a reduced level of endogenous plectin resulted in inhibited cell invasion in the HNSCC cell line.

3.4. Phosphorylated Erk 1/2 decreases in plectin knockdown HNSCC cells

What is the mechanism of the inhibition of migration and invasion in plectin knockdown HNSCC cells? A previous report by Osmanagic-Myers et al. showed that plectin $^{-/-}$ keratinocytes migrate faster than plectin $^{+/+}$ cells, which is contrary to our results [15]. They showed that basal phosphorylation

Table 2 – Protein increased in HNSCC.

Protein increased in tumor tissue												
Spot no. ^{a)}	Database accession no.	Unit ProtKB accession no.	Protein name	Average mass	Score	Seq. cov. (%) ^{b)}	MS/MS (unique) ^{c)}	Fold increase	Standard deviation	t-test	Pretension mass	References ^{e)}
T1	gi-4507651	P67936	Tropomyosin 4	28,504	147	14	4	1.98	0.95	<0.001	33,000	(1)
T2	gi-187302	P31947	Epithelial cell marker protein 1	27,759	152	19	5	2.57	2.26	<0.001	31,000	(1,2)
T3	gi-49119653	P63104	YWHAZ protein	29,929	191	18	4	1.50	0.30	<0.001	31,000	(2)
T4	gi-16507237	P11142	Heat shock 70 kD protein 5	72,288	683	28	18	1.57	0.78	0.096	85,000	(3)
T5	gi-1199487	P50454	Collagen binding protein 2	46,506	245	22	8	4.54	1.95	<0.001	46,000	
T6	gi-45604447	P22626	Heterogeneous nuclear ribonucleoprotein A2/B1	35,984	145	14	3	2.01	1.00	0.015	34,000	
T7	gi-4505591	Q06830	Perox iredoxin 1	22,096	116	22	4	1.85	0.81	<0.001	23,000	(4)
T8	gi-62738525	P36952	Serpin	42,615	328	26	8	1.65	1.04	0.042	40,000	(5)
T9	gi-4503471	P68104	Eukaryotic translation elongation factor 1 alpha 1	50,109	215	11	5	5.39	2.79	<0.001	47,000	
T10	gi-42544159	Q92598	Heat shock 105 kD	96,804	329	10	8	1.52	0.56	0.028	110,000	
T11	gi-4504165	P06396	Gelsolin	85,644	286	10	6	1.52	0.41	0.018	90,000	(7)
T12	gi-10863945	P13010	ATP-dependent DNA helicase II	82,652	201	12	7	2.56	1.64	<0.001	880,000	
T13	gi-4507115	Q16658	Fascin 1	54,496	104	7	3	2.41	2.07	0.004	51,000	(8)
T14	gi-5031573	P61158	Actin-related protein 3	47,341	148	11	4	4.90	4.66	0.008	48,000	(8)
T15	gi-4503445	P19971	Endothelial cell growth factor 1 precursor	49,924	486	25	12	2.67	1.41	0.014	51,000	
T16	gi-30130	P50454	Collagen	46,238	97	6	2	3.25	2.74	0.013	47,000	(9)
T17	gi-75517570	P09651	HNRPA 1 protein	29,368	306	33	7	2.50	1.71	0.025	33,000	
T18	gi-181967	P68104	Elongation factor 1-alpha	35,205	52	3	1 ^{d)}	1.93	1.00	0.075	32,000	
T19	gi-49168580	P40926	MDH2	35,537	613	46	15	3.02	4.53	0.031	34,000	
T20	gi-4557032	P07195	Lactate dehydrogenase B	36,615	213	20	6	1.53	0.54	0.006	33,000	
T21	gi-18645167	P07355	Annexin A2	38,552	177	18	5	4.57	5.05	<0.001	3200	(10)
T22	gi-37724561	P63244	Lung cancer oncogene 7	37,865	181	11	3	3.03	4.85	0.016	32,000	
T23	gi-48255905	Q01995	Transgelin	22,596	151	29	5	2.60	1.55	<0.001	25,000	
T24	gi-4504517	P04792	Heat shock 27 kDa protein 1	22,768	261	31	5	1.57	0.34	<0.001	30,000	(1)
T25	gi-47939618	P09651	Heterogeneous nuclear ribonucleoprotein A1	34,159	202	13	4	6.48	7.41	<0.001	32,000	
T26	gi-31542947	P10809	Chaperonin	61,016	496	34	16	2.08	1.70	0.01	62,000	(11)
T27	gi-31542947	P30101	ER0-60 protease	56,761	427	23	14	1.83	0.87	0.044	60,000	
T28	gi-320200	Q00839	Scaffold attachment factor A	90,423	427	11	8	2.59	1.37	0.009	120,000	
T29	gi-63252900	P09493	Tropomyosin 1 alpha chain	32,856	205	20	7	2.59	1.80	0.025	33,000	(11)
T30	gi-68533131	Q4LE33	TNC variant protein	244,248	2222	20	41	3.98	1.91	0.009	250,000	
T31	gi-41322908	Q15149	Plectin 1	513,393	503	5	20	1.69	0.64	0.035	30,000	

^a Spot numbers refer to those in Fig. 1.

^b Amino acid sequence coverage for the identified protein.

^c Number of peptide fragments of a protein that yielded informative MS/MS data (number of unique peptide). The minimum criterion of the probability-based MASCOT/MOWSE score was set with 5% as the significant threshold level

^d MS/MS spectrum refers to Fig. S2.

^e The references are listed in Supplementary Information 3.

Table 3 – Protein decreased in HNSCC.

Protein decreased in tumor tissue												
Spot no. ^{a)}	Database accession no.	Unit ProtKB accession no.	Protein name	Average mass	Score	Seq. cov. (%) ^{b)}	MS/MS (unique) ^{c)}	Fold decrease	Standard deviation	t-test	Pretension mass	References ^{d)}
N1	gi-4502101	P04083	Annexin I	38,690	336	21	8	7.49	7.59	<0.001	39,000	(12)
N2	gi-4557871	P02787	Transferrin	77,000	259	11	7	5.13	3.07	<0.001	83,000	(13)
N3	gi-177831	P01009	Alpha-1-antitrypsin	46,677	252	21	8	6.18	4.08	<0.001	66,000	(2)
N4	gi-7706635	Q9UBG3	Cornulin	53,502	244	18	5	7.10	6.06	<0.001	66,000	(3)
N5	gi-179106	Q13813	Nonerythroid alpha-spectrin	284,107	648	11	24	1.81	0.81	0.014	220,000	
N6	gi-2992541	O60437	Periplakin	204,580	975	22	35	4.32	3.02	<0.001	182,000	
N7	gi-27436946	P02545	Lamin A/C	74,095	450	21	13	2.16	1.18	<0.001	81,000	(14)
N8	gi-119589476	P01024	Complement component 3	143,619	347	9	9	2.81	1.83	0.005	70,000	
N9	gi-37267	P29401	Transketolase	67,751	169	11	6	4.05	4.11	0.016	68,000	
N10	gi-340219	P08670	Vimentin	53,681	382	29	14	2.15	1.96	0.005	48,000	
N11	gi-40647126	P01860	Anti-HIV-1 gp120 immunoglobulin	22,899	54	11	2	4.96	5.15	0.014	52,000	(1,2)
N12	gi-12798841	P25705	ATP synthase	59,671	100	6	3	5.18	5.32	0.038	53,000	(11)
N13	gi-297412	P35237	Thrombin inhibitor	42,559	69	6	2	2.61	1.75	0.008	43,000	
N14	gi-178775	P02647	Propolipoprotein	28,944	187	21	5	4.58	2.14	<0.001	25,000	
N15	gi-4504517	P04792	Heat shock 27 kDa protein 1	22,768	279	33	7	1.72	0.62	<0.001	31,000	(1)
N16	gi-149673887	P01834	Immunoglobulin lights chain	23,331	109	17	2	2.44	1.15	<0.001	30,000	(1)
N17	gi-4502517	P00915	Carbonic anhydrase I	28,852	249	31	7	2.41	1.25	0.016	32,000	(2)
N18	gi-34234	P08865	Laminin-binding protein	31,774	141	14	3	5.16	10.01	0.004	44,000	(2)
N19	gi-87196339	P12109	Collagen type VI, alpha 1 precursor	108,462	319	10	9	3.52	5.06	<0.001	120,000	
N20	gi-41350923	P12110	Collagen type VI, alpha 2	108,539	151	5	4	3.43	4.91	0.026	120,000	
N21	gi-1147813	P15924	Desmoplakin I	331,571	190	3	7	2.45	2.08	0.043	220,000	(15)
N22	gi-4503483	P13639	Eukaryotic translation elongation factor 2	95,277	91	3	3	4.23	5.40	0.042	97,000	(15)
N23	gi-136066	P060174	Triosephosphate isomerase	26,609	432	38	7	2.58	1.60	<0.001	30,000	
N24	gi-14194715	Q92817	Envoplakin	231,477	110	1	2	2.52	2.24	0.007	20,000	
N25	gi-860986	P07237	Protein disulfide-isomerase	56,644	141	8	5	2.10	1.63	0.061	47,000	(13)
N26	gi-13489087	P30740	Serine proteinase inhibitor	42,715	427	34	11	3.37	2.84	<0.001	41,000	(14)
N27	gi-4507877	P18206	Vinculin	116,649	177	6	7	1.93	0.71	<0.001	100,000	
N28	gi-55743104	P12111	Alpha 3 type VI collagen precursor	321,987	421	5	17	2.05	1.55	0.013	190,000	
N29	gi-5729877	P11142	Heat shock 70 kDa protein 8	70,854	156	7	4	4.76	4.74	0.004	62,000	(3)
N30	gi-386785	P08107	Heat shock protein	69,825	462	19	12	3.14	2.46	0.005	61,000	
N31	gi-157671915	Q6ZNG6	Guanylate binding protein 6	72,381	65	3	2	2.85	1.71	0.002	61,000	(3)
N32	gi-61743954	Q09666	AHNAK nucleoprotein	628,699	323	3	15	2.44	1.98	0.014	260,000	
N33	gi-124731	P07476	AHNAK nucleoprotein	68,427	218	15	7	2.16	1.35	0.003	115,000	
N34	gi-492570004	P13928	Annexin A8	36,872	69	6	2	3.66	2.71	0.035	35,000	(2)

^a Spot numbers refer to those in Fig. 1.

^b Amino acid sequence coverage for the identified protein.

^c Number of peptide fragments of a protein that yielded informative MS/MS data (number of unique peptide). The minimum criterion of the probability-based MASCOT/MOWSE score was set with 5% as the significant threshold level.

^d The references are listed in Supplementary Information 3.

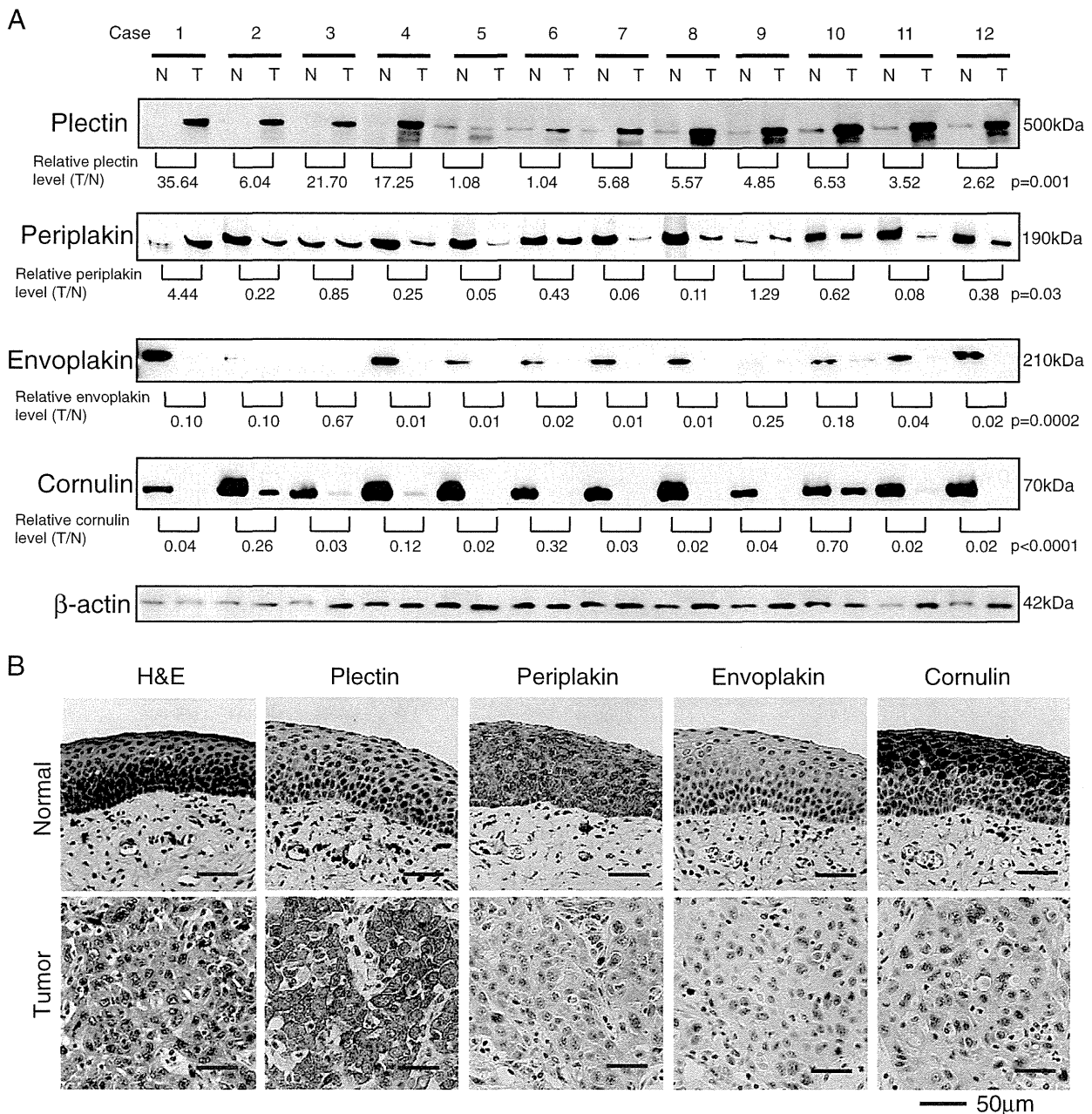


Fig. 2 – Validation of differentially expressed proteins in HNSCC tissues by Western blotting and immunohistochemistry. A, Western blots of total protein lysates prepared from twelve matched samples of tumor (T) and adjacent non-tumor tissue (N) with anti-plectin, anti-periplakin, anti-envoplakin, anti-cornulin, and anti- β -actin antibodies (loading control). The intensity of each band was measured with TotalLab™ 1D software (Nonlinear Dynamics, Newcastle upon Tyne, UK), and protein levels were calculated between tumor and non-tumor tissue, normalized with β -actin. The difference in protein expressions between (T) and (N) was assessed by Student's t-test for unpaired values. B, Immunohistochemical analysis of paraffin-embedded normal epithelia and cancer tissues with plectin, periplakin, envoplakin, and cornulin antibodies. HE: hematoxylin and eosin.

of Erk 1/2 kinases was significantly elevated in plectin-/-keratinocytes and was the cause of the faster migration of plectin-deficient cells. Upregulation of Erk 1/2 kinases has been shown to play a role in tumor invasion by inducing EMT [16], or by promoting the degradation of extracellular matrix proteins through induction of MMPs [17,18]. Furthermore,

overexpression of N-cadherin induces cell migration in vitro and invasion and metastasis in vivo by an Erk 1/2-dependent mechanism [19,20]; therefore, we investigated Erk 1/2 activities in plectin knockdown HNSCC cells. Surprisingly, phosphorylated Erk 1/2 was significantly decreased in plectin siRNA-treated cells as compared with control siRNA-treated

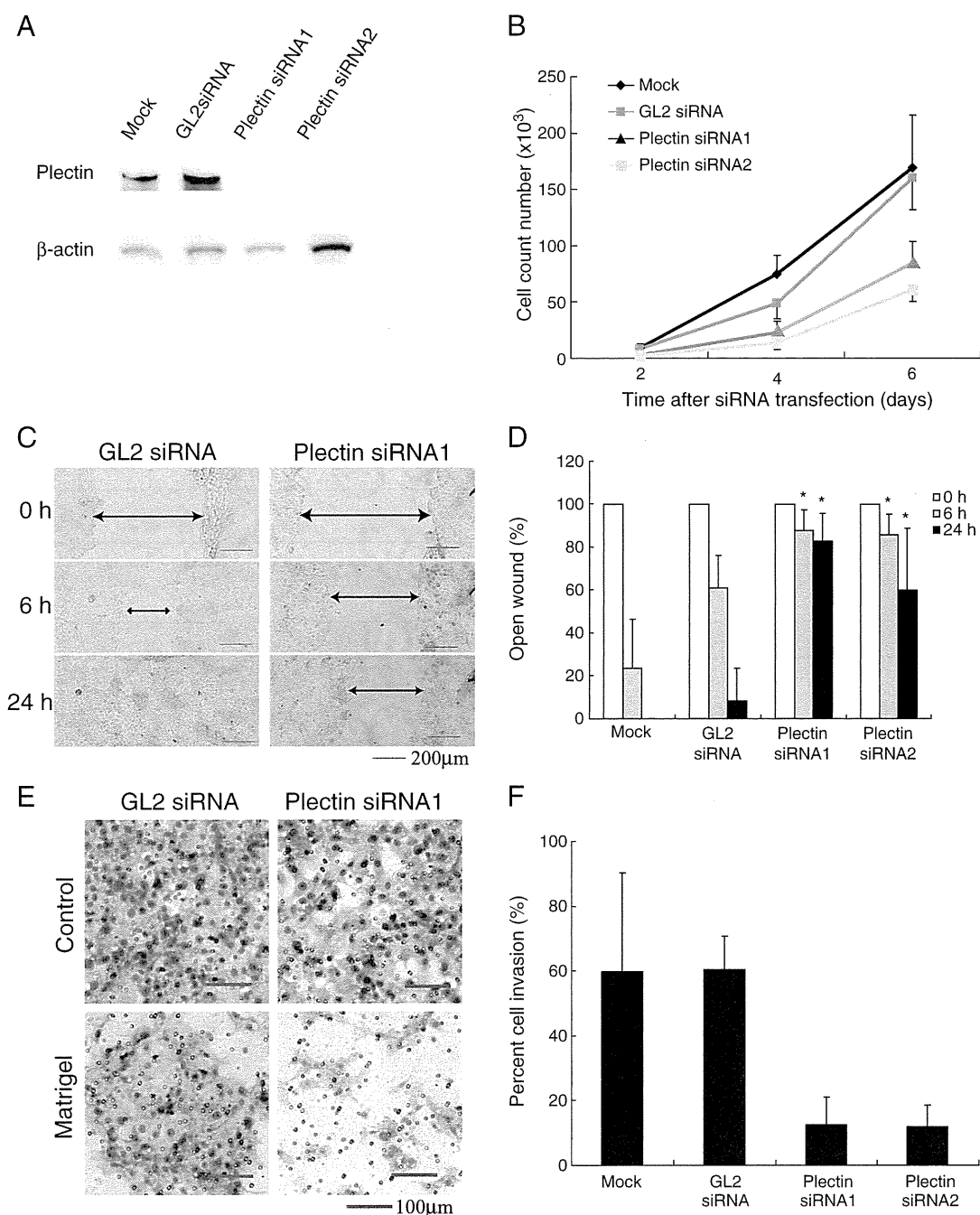


Fig. 3 – Suppression of plectin inhibits the cell proliferation, migration, and invasion of HNSCC cells. A, Expression of plectin in D562 cells was determined by Western blotting 24 hrs after plectin siRNA treatment. GL2 siRNA was used as a control. β -actin was used as a control for internal protein loading. B, Knockdown of plectin expression by siRNA significantly suppresses the proliferation of D562 cells. Cells were transfected with Mock, GL2siRNA, plectin siRNA1 and -2. Cells were cultured for the indicated time. *, $P < 0.05$, control siRNA compared with siRNAs. C and D, Suppression of plectin decreased cancer cell migration. Cells were scratched with a pipette tip and migration toward the wounded area was observed. *, $P < 0.05$, control siRNA compared with plectin siRNA. E, Suppression of plectin decreased cancer cell invasion. Representative images show cells that passed through the control chamber or Matrigel when transfected with control and plectin siRNA1. F, Three randomly selected fields were photographed and the number of invaded cells was counted. Percent cell invasion was calculated as the mean number of cells passing through a Matrigel insert membrane relative to passing through a control insert membrane. *, $P < 0.05$, control siRNA compared with plectin siRNAs.

cells (Fig. 4A,B). Total Erk 1/2 level was also slightly decreased in plectin siRNA-treated cells (Fig. 4A,B). Although the relationship between plectin expression and Erk 1/2 activity

remains obscure, these results suggest that the inhibition of migration and invasion in plectin knockdown cells may be associated with downregulation of Erk 1/2 kinase activities,

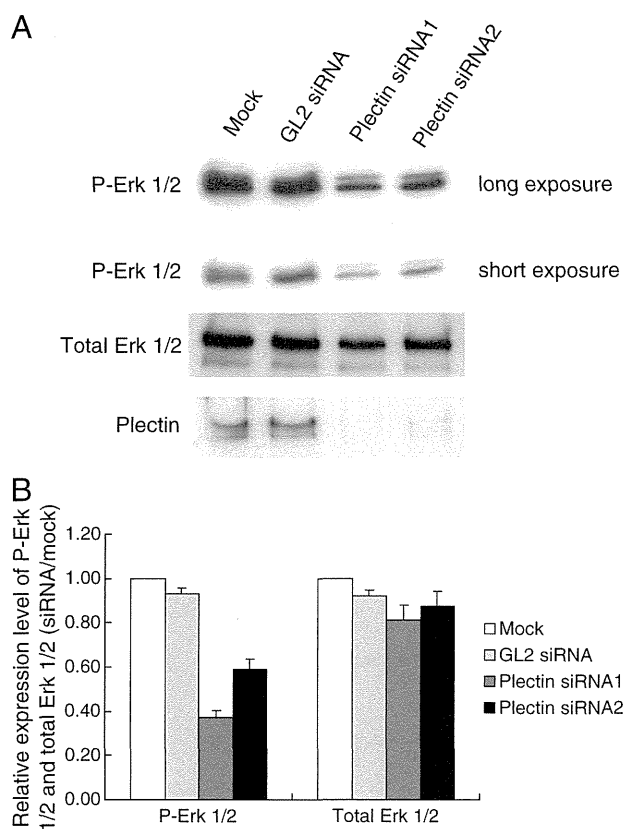


Fig. 4 – Suppression of plectin attenuates phosphorylated Erk 1/2 level. A. Expression of phosphorylated Erk 1/2 (P-Erk 1/2) and total Erk 1/2 in D562 cells was examined by Western blotting 24 hrs after plectin siRNA treatment. GL2 siRNA was used as a control. B. The intensity of P-Erk 1/2 and total Erk 1/2 bands in siRNA-treated cells were measured with TotalLab TL120 and the relative expression levels of each protein as compared with mock treatment are shown.

which explains the different migration behavior between plectin-deficient keratinocytes and HNSCC cells.

3.5. Plectin is a potential prognostic marker of HNSCC

The above experiments strongly imply that upregulation of plectin is involved in metastasis and, as a result, the malignant potential of HNSCC. For this reason, we next examined whether plectin is associated with the prognosis of HNSCC patients. Immunohistochemistry of paraffin-embedded tissues obtained from 62 HNSCC patients was performed using anti-plectin antibody and its expression level was judged on a scale of 0 to 3+: 0, no staining; 1+, weak staining; 2+, moderate staining; 3+, strong staining (Fig. S1A). For statistical analysis, scores of 0 and 1+ were rated as low expression, and scores of 2+ and 3+ were rated as high expression. Survival curves were calculated by the Kaplan-Meier method and analyzed using the log-rank test. Strikingly, the survival rate of patients with a higher plectin level ($\geq 2+$) was significantly decreased ($P < 0.001$) (Fig. 5A).

To test if the correlation of the survival rate and plectin expression level is comparable to other well-known prognosis markers of HNSCC, we analyzed the survival rate according to

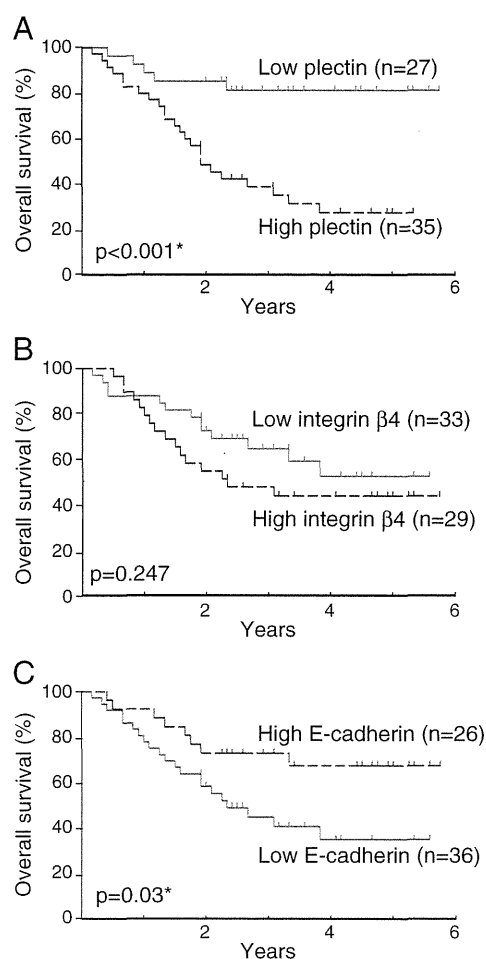


Fig. 5 – Kaplan-Meier survival curves according to expression levels of plectin ($P < 0.001$; A), integrin $\beta 4$ ($P = 0.247$; B), and E-cadherin ($P = 0.03$; C) in HNSCC.

the expression levels of integrin $\beta 4$ and E-cadherin, which were previously reported to correlate with the clinical outcomes of HNSCC patients [21–24]. Integrin $\beta 4$ and E-cadherin expression levels were examined by IHC and judged on a scale of 0 to 3+ according to the intensity of staining (Fig. S1B, C). The results showed that a high expression of integrin $\beta 4$ was not correlated with the poor prognosis of HNSCC patients ($P = 0.247$, log-rank test) (Fig. 5B). Moreover, although a low expression of E-cadherin was correlated with the poor prognosis of HNSCC patients ($P = 0.03$, log-rank test), its P value was much higher than that of statistical analysis by plectin expression (Fig. 5C). These results strongly indicate that plectin is a promising prognostic marker for HNSCC.

We further investigated whether the expression levels of plectin, integrin $\beta 4$, and E-cadherin in cancer cells correlated with the clinical outcomes of HNSCC patients (Table 4). There was no significant correlation between plectin, integrin $\beta 4$, or E-cadherin expression and gender, age, location of tumors, histology, clinical stage, or local recurrence; significant correlation was only observed between the plectin expression level and with or without distant metastasis in HNSCC patients. This result further supports the idea that plectin is involved in the metastasis and malignant potential of HNSCC.

Table 4 – Characteristics of 62 HNSCC patients in IHC analysis.

	Total (62)	Plectin-IHC expression		P	Integrin β 4-IHC expression		P	E-cadherin-IHC expression		P
		Low (27)	High (35)		Low (33)	High (29)		Low (36)	High (26)	
Gender										
Male	50	21	29		27	23		31	19	
Female	12	6	6	NS	6	6	NS	5	7	NS
Age										
≥ 60	39	17	22		23	16		22	17	
<60	23	10	13	NS	10	13	NS	14	9	NS
Location of tumors										
Oral	23	11	12		14	9		12	11	
Oropharynx	2	1	1		1	1		2	0	
Hypopharynx	29	12	17		12	17		16	13	
Larynx	6	2	4		5	1		4	2	
Salivary gland	2	1	1	NS	1	1	NS	2	0	NS
Histology										
Well	18	10	8		12	6		7	11	
Moderate	38	14	24		19	20		26	12	
Poorly	6	3	3	NS	3	3	NS	3	3	NS
UICC-Stage										
I-III	17	10	7		12	5		9	8	
VI	45	17	28	NS	21	24	NS	27	18	NS
Local recurrence										
Yes	11	3	8		5	6		8	3	
No	51	24	27	NS	28	23	NS	28	23	NS
Distant metastasis										
Yes	25	7	18		14	11		15	10	
No	37	20	17	0.042	19	18	NS	21	16	NS

*Significance value $P < 0.05$

NS, not significant

3.6. Plectin is an independent prognostic marker of HNSCC

Finally, we analyzed whether the plectin expression level was an independent clinical outcome factor in HNSCC patients. On univariate analysis, the clinical stage, plectin expression and E-cadherin expression were correlated with the overall survival time (Table 5, left). Furthermore, among these factors, the clinical stage (hazard ratio, 3.403; 95% confidence interval, 1.024–11.309, $P=0.0456$), plectin expression (hazard ratio, 5.519; 95% confidence interval, 1.653–18.433, $P=0.0055$) and E-cadherin expression (hazard ratio, 0.418; 95% confidence interval, 0.184–0.950, $P=0.0373$) were independent clinical outcome factors on multivariate analysis (Table 5, right). The data strongly suggested that plectin alone might be a prognostic biomarker of HNSCC.

4. Discussion

In this study, we searched for proteins that could serve as biomarkers for cancer diagnosis, prognosis, and therapeutic targets in HNSCC by agarose 2D-DIGE using surgically resected clinical specimens, and succeeded in identifying and validating several proteins that were differentially expressed in HNSCC. Among them, plectin was significantly overexpressed in HNSCC. Strikingly, HNSCC patients with a high plectin level suffered recurrence more frequently than those with a low plectin expression; as a result, the survival rate of patients with a high plectin level significantly declined. Furthermore, functional

studies implied that a decreased expression of plectin suppresses the proliferation, migration and invasion of HNSCC cells. This is probably due to the downregulation of Erk 1/2 kinase activity in the cells, although whether it is caused by attenuated expression of plectin or by some other mechanism is unclear. These results provided evidence that plectin is a novel and promising prognostic biomarker of HNSCC.

Our results indicated that patients with a higher plectin level showed a much lower overall survival rate than those with a lower plectin level (Fig. 5). This difference in the survival rate is probably related with the higher metastatic potential of HNSCC with an increased plectin level (Table 4), which was supported by our experimental data and previous studies indicating that plectin is involved in cell migration and invasion. We showed that the suppression of endogenous plectin inhibited the migration and invasion of HNSCC cells (Fig. 3). In other studies, ablation of plectin impaired the migration of MCF-7 (human breast adenocarcinoma cell line) epithelial sheets [25]. Moreover, plectin interacts with the integrin β 4, a receptor for laminins, which is a major component of the epidermal basement membrane [26]. Integrin β 4 has been reported to associate with cell migration and invasion [12–14]. Furthermore, its high expression is associated with poor prognosis in a variety of human cancers [27,28]. Thus, overexpression of plectin might contribute to cell migration and invasion in HNSCC cells through its association with integrin β 4.

On the other hand, Osmanagic-Myers et al. showed opposite results that suppression of plectin accelerates the

Table 5 – Prognostic factors by univariate and multivariate analysis.

Variables	Univariate analysis		Multivariate analysis	
	Hazard ratio 95% confidence interval	P	Hazard ratio 95% confidence interval	P
Gender (Male/Female)	0.608 (0.211-1.750)	0.3560		
Age (<60/≥60)	1.087 (0.505-2.340)	0.8312		
Location of tumors (larynx, pharynx/others)	0.494 (0.218-1.122)	0.0921		
WHO histological type (poor-moderate/well)	2.402 (0.913-6.325)	0.0759		
Clinical stage (I-III/VI)	4.471 (1.351-14.801)	0.0142*	3.403 (1.024-11.309)	0.0456*
Plectin (high/low)	6.359 (1.918-21.078)	0.0025*	5.519 (1.653-18.433)	0.0055*
Integrin β4 (high/low)	1.538 (0.739-3.204)	0.2498		
E-cadherin (low/high)	0.416 (0.184-0.942)	0.0355*	0.418 (0.184-0.950)	0.0373*

*Significance value $P < 0.05$.

migration of keratinocytes [15]. How can this contradiction be explained? In keratinocytes, a MAP kinase Erk 1/2, which is known to positively regulate keratinocyte migration [29], was activated when plectin was depleted. This result was in marked contrast with our result that phosphorylated Erk 1/2 was down-regulated in plectin-deficient HNSCC cells. Ding et al. also reported that knockdown of plectin with RNA interference (RNAi) attenuates the activation of Erk1/2 in HEK293 cells [30]. The above results suggest that the effect on migration in plectin-deficient cells depends on the different response of Erk 1/2 activity to the alteration of plectin expression in each cell line.

Another mechanism of plectin-mediated migration and invasion might be its association with CXCR4 through the plectin N-terminal domain [30]. CXCR4 is the chemokine receptor for SDF-1 and has been reported to not only be expressed in a wide variety of carcinomas [31-34], but to be correlated with distant metastasis and poor prognosis in HNSCC [35]. Moreover, SDF-1/CXCR4 interaction is known to be involved in cell proliferation, migration, and invasion evoked through Erk 1/2 in several cancer cells [36-40]. This interaction and signal system may be related to the poor prognosis of HNSCC with high plectin expression.

We also observed that suppression of plectin inhibited the proliferation of HNSCC cells (Fig. 3), which suggests that plectin is involved in cancer development. In fact, overexpression of plectin has been previously reported in prostate cancer, colorectal cancer, and pancreatic cancer [41-43], although the precise mechanism of how plectin overexpression is related to cancer development is not clear. Plectin is a large protein that links intermediate filaments to microtubules and microfilaments, anchoring the cytoskeleton to the plasma and nuclear membranes [44,45]. It also plays important roles in signal transduction, is involved in Rho/Rac/Cdc42 signaling cascades [46] and is an early substrate for caspase 8 following apoptosis induction by CD95 or tumor necrosis factor receptor [47]. Thus, upregulation of plectin in HNSCC may have an impact on the signaling pathways that regulate cell migration and apoptosis.

Postoperative adjuvant therapy for HNSCC is now applied on the basis of the results of pathological examination of the surgical specimens (multiple lymph node metastases, extralymphatic invasion, a positive stump after microscopic

resection). Efficacy of chemoradiotherapy as compared with radiotherapy alone was evaluated in two randomized trials conducted in Europe (European Organization Research and Treatment of Cancer; EORTC) and the United States (Radiation Therapy Oncology Group; RTOG) [48,49]. Although EORTC study revealed that chemoradiotherapy significantly increased overall survival without a high incidence of adverse effects, RTOG trial showed not only a marginal improvement of overall survival but significant increase in severe adverse effects with chemoradiotherapy. Inconsistencies emerged from these reports emphasizes an intense need to define more precise boundaries demarcating risk levels for the decision-making processes of adjuvant treatment [50]. Therefore, while chemotherapy combined with radiotherapy are effective as postoperative adjuvant therapies for HNSCC, better biomarkers to select patients with proper indications are necessary to practically improve the clinical outcome of the patients and/or prevent a risk of adverse effects of the treatment. In this regard, plectin could be a useful biomarker for more effective postoperative treatment options, such as radiation alone for patients with a low plectin level and radiation in combination with chemotherapy for patients with a high plectin level.

In summary, we identified plectin as a promising prognostic biomarker in HNSCC, which could make significant contributions to the prediction of HNSCC patient prognosis and might improve their clinical outcome through postoperative follow-up and additional therapy. Further investigation is needed to uncover the mechanisms responsible for plectin-mediated development of HNSCC.

Supplementary materials related to this article can be found online at doi:10.1016/j.jprot.2011.12.018.

Disclosure

The authors declare no conflicts of interest.

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