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# Initiation of malignancy by duodenal contents reflux and the role of ezrin in developing esophageal squamous cell carcinoma

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**Gastroesophageal reflux has recently been implicated as a causative factor in upper aerodigestive tract carcinogenesis. Esophageal squamous cell carcinomas (ESCCs) have developed in duodenal-content reflux animals without any known carcinogen present. We established a cell line, designated ESCC-DR, from a thoracic metastatic tumor in a reflux animal. To gain insight into the genomic alterations associated with duodenal content reflux-induced carcinogenesis, we first performed comparative genomic hybridization using an Agilent rat 244K array in ESCC-DR and identified many chromosomal gains and losses. Of the many genes identified, we detected an interesting ezrin amplicon that has been recently reported in human ESCC. Ezrin, which cross-links the cytoskeleton and plasma membrane, is involved in the growth and metastatic potential of cancer cells. Overexpression of ezrin protein in ESCC-DR was confirmed by Western blotting. We also compared ezrin protein expression levels and patterns in hyperplastic, dysplastic, ESCC, and metastatic sites developed in two distinct reflux models using immunohistochemistry. Immunohistochemical staining of ezrin revealed overexpression in the nucleus, and the cytoplasm as well as plasma membrane of ESCC cells. Phosphorylated ERM (ezrin, radixin, moesin) was expressed at the leading edge, or invasive front, of larger metastatic sites. Taken together, duodenal reflux has a great potential for initiating malignancy, and thus likely plays a role in development of ESCC. Ezrin probably influences the growth and invasiveness of ESCC cells, and phosphorylation is only required in metastatic behavior of tumor cells at the leading edge and invasive front. (*Cancer Sci* 2010; 101: 624–630)**

Esophageal cancer is the eighth most-common cancer and the sixth most-common cause of cancer-related mortality in the world.[1] This malignancy exists as esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), which each have distinct etiological and pathological characteristics. ESCC is generally associated with smoking and alcohol consumption.[2–4] In addition, individuals with long-standing and severe symptoms of reflux display odds ratios of 43.5 for EAC and 1.1 for ESCC.[5] However, recent studies have demonstrated that refluxed duodenal contents cause esophageal carcinoma in rats without exposure to carcinogens.[6–9] Furthermore, we previously established an ESCC cell line, designated ESCC-DR, from a thoracic metastatic tumor in a rat duodenal-content reflux model at the 60th post-operative week.[9] To explore the genome-wide chromosomal imbalance associated with duodenal content reflux-induced ESCC, we first used a high-resolution oligonucleotide comparative genomic hybridization (CGH) array to assess the genome-wide chromosomal imbalance in the ESCC-DR cell line. The CGH array analysis was conducted using an Agilent 244K whole-genome array (Agilent Technologies, Santa Clara, CA, USA), and we identified many chromosomal gains and losses. We then noticed an interesting ezrin amplicon, which has been recently described in ESCC.[10,11] Ezrin was initially isolated as a cytoskeletal component of intestinal microvilli and a substrate for tyrosine kinase.[12] Ezrin is a member of the ERM (ezrin, radixin, moesin) protein family and links F-actin to the cell membrane following phosphorylation.[13–16] This linker function makes ezrin essential for many fundamental cellular processes, including the determination of cell shape, polarity and surface structure, cell adhesion, motility, cytokinesis, phagocytosis, and integration of membrane transport through signalling pathways.[17–20] ERM proteins exist in the cytosol in an inactive state and upon activation, undergo a conformational change that allows binding to both plasma membrane proteins and actin filaments of the cytoskeleton,[21] resulting in a physical and signal transduction link between the plasma membrane and cytoskeleton. ERM protein activity is regulated by conformation. When inactive, the N- and C-terminal domains of the protein self-associate, masking the actin binding site.[22] Phosphatidylinositol 4,5-bisphosphate ($PIP_2$) binding to an unidentified site in the N-terminal domain brings about activation, with sequential phosphorylation of a specific C-terminal threonine that stabilizes ERM proteins in their active conformation, probably by preventing interaction of the N- and C-terminal domains.[23–26] Moreover, ezrin expression has been linked to poor survival in several cancers, including carcinomas of the breast, colon, endometrium, and ovary; cutaneous and uveal melanomas; and brain tumours and soft tissue sarcomas.[27–34]

In this study, we present data indicating that duodenal reflux has a great potential for initiating malignancy, and we examined the role of ezrin and pERM in ESCC and its development using various lesions developed in two distinct duodenal content-reflux models and the ESCC-DR cell line.

## Materials and Methods

All procedures complied with the ethical guidelines for animal experimentation and the care and use of laboratory animals at Shiga University of Medical Science, Japan.

**ESCC-DR cell culture.** ESCC-DR is an ESCC cell line established in our department. This cell line originated from a thoracic metastatic tumor developed in a duodenal content-reflux model at the 60th post-operative week.[9] ESCC-DR were grown and maintained in Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Kyoto, Japan) supplemented with 1% antibiotic-antimycotic solution (Gibco, Grand Island, NY, USA)

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and 10% fetal bovine serum (FBS) in a humidified incubator containing 5% $CO_2$ at 37°C.[9]

**DNA extraction from normal esophageal epithelium and ESCC-DR cells.** As a normal control, 8-week-old male Wistar rats were sacrificed using diethyl ether; esophagi were excised, and DNA was extracted from the esophageal epithelium and the ESCC-DR cells. DNA was isolated by a standard procedure involving enzymatic digestion of protein and RNA followed by extraction with the Qiagen DNA extraction kit (Qiagen, Valencia, CA, USA). DNA concentration and purity were determined using a NanoDrop 100ND-1000 Spectrometer (Thermo Scientific, Rockford, IL, USA).

**CGH array profiling.** DNA was hybridized to an Agilent 244K whole-genome array at the Dragon Genomics Center, Takara Bio (Shiga, Japan). For each sample, 500 ng of DNA was fragmented via double enzymatic digestion (AluI + RsaI) and hybridization. ESCC-DR and control DNA from normal rat esophageal epithelium were labeled by random priming with CY5-dUTP and CY3-dUTP, respectively, and hybridized at 65°C for 40 h. The chips were scanned on an Agilent G2565BA DNA microarray scanner, and image analysis was performed using the Feature Extraction software version 9.5 (Agilent Technologies). Feature extraction was used for the fluorescent signal acquisition from the scans. Values were normalized using the linear normalization method available in the Feature Extraction software version 9.5 with a default value for all parameters. Raw copy number ratio data were transferred to the CGH Analytics software version 3.5.14 (Agilent Technologies) for further analysis. The ADM-2 algorithm of the CGH Analytics software version 3.5.14 was used to identify DNA copy number anomalies at the probe level.

**Western blot analyses of ezrin and phosphorylated ERM in ESCC-DR cells.** Protein was extracted from ESCC-DR cells and normal rat esophageal epithelium. We also used the protein extracted from the lung tissue of a male Wistar rat as a positive control for ezrin and pERM. Western blot analysis was performed using a rabbit anti-ezrin polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:1000, a rabbit anti-phospho-ezrin (Thr567)/radixin (Thr564)/moesin (Thr558) (phosphorylated ERM) monoclonal antibody (Cell

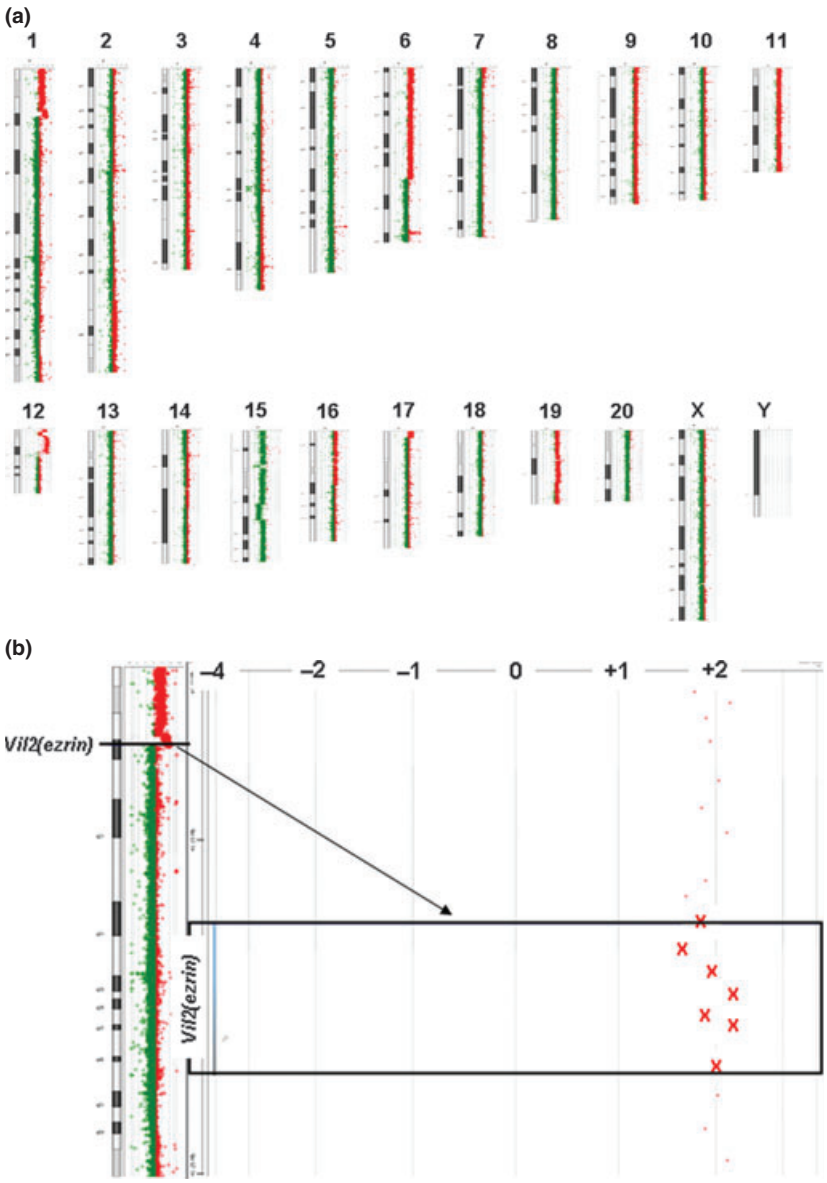


**Fig. 1.** Representative comparative genomic hybridization (CGH) profile for the esophageal squamous cell carcinoma (ESCC)-DR cells. (a) CGH profile, (b) a section of chromosome 1. (a) Average $\log_2$ ratios are plotted for all clones based on chromosome position. Detection of many gains and losses suggests that the ESCC-DR cell line is genetically quite unstable (green, decrease; red, increase). (b) The $\log_2$ ratios of CGH enrichment detected by the genomic segmentation algorithm are represented. Seven ezrin probes encoded by *vil2* are located in 1q11. There was an approximate 2-fold change in ESCC-DR cells compared to normal esophageal epithelium.

Signaling Technology) at 1:1000 and a mouse monoclonal anti-β-actin antibody (C4, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:1000.

**Western blot of ezrin and pERM obtained from fractionated ESCC-DR cells.** Crude membrane fractions from ESCC-DR cells were prepared as described previously.[35] ESCC-DR cells cultured in a 100-mm dish were washed with PBS, and incubated with 2 mL of low ionic salt buffer composed of 0.5 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.4) at 0°C for 10 min. After adding 1 mM phenylmethylsulfonyl fluoride and 0.09 units/mL aprotinin, cells were homogenized with a Dounce homogenizer, and the homogenate was diluted with an equal volume of 500 mM sucrose and 10 mM Tris-HCl, pH 7.4. The homogenate was centrifuged at 800$g$ for 10 min to precipitate the fraction containing nuclei. The supernatant was centrifuged at 10 000$g$ for 30 min to precipitate the fraction containing mitochondria and lysosomes. The supernatant was further centrifuged at 100 000$g$ for 90 min to precipitate the microsomal and plasma membrane fraction. The pellet was resuspended in 250 mM sucrose and 5 mM Tris-HCl (pH 7.4).

**Immunohistochemical staining of ezrin and pERM in ESCC-DR cells.** Immunohistochemical staining was performed using a modified streptavidin-peroxidase-conjugated method based on the poly-horseradish peroxidase (HRP) antimouse/rabbit IgG detection system of the Polymer Detection System (Histofine MAX-PO [MULTI]; Nichirei, Tokyo, Japan).

**Animal samples and histological examination.** We selected six ESCCs cases, which had been developed in other previous studies using the following distinct reflux animal models.[6–9] One model was reported in 2003. The model was anastomosed side-by-side between the upper jejunum and esophago-gastric junction without gastrectomy.[6,7] The other model was reported in 2007.[8,9] This model was anastomosed end-to-side between the cut end of the esophagus and upper jejunum after transsection of the esophago-gastric junction. Two of the six ESCCs were developed in the former model, which survived until 70 and 100 weeks; and four of the six ESCCs were developed in the latter model, one at 30 and three at 60 weeks postoperatively. In one of the cases, ESCC advanced with metastases to the lungs and lymph nodes .[9] The ESCCs and metastases in the lymph nodes and lung were used as formalin-fixed and paraffin-embedded specimens. We also investigated ezrin and pERM distribution in the progression from normal epithelium to invasive carcinoma of the esophagus using various adjacent hyperplastic and dysplastic lesions that were developed in the above six ESCC cases. Esophageal tissues from five sham-operated rats were used as controls.

**Immunohistochemistry with paraffin sections.** Dewaxed sections were examined using a mouse anti-ezrin antibody (Clone 3C12; Invitrogen, Carlsbad, CA, USA) at 1:200 and an anti-phosphorylated ERM antibody (Cell Signaling Technology) at 1:100. Immunohistochemical procedures were performed using the Polymer Detection kit (Histofine MAX-PO [MULTI]; Nichirei), followed by detection/visualization with 3,3′-diaminobenzidine tetrahydrochloride (DAB; Histofine).

## Results

**Chromosomal and genetic instability.** The CGH array data in the ESCC-DR revealed chromosomal gains on 1p11-q11, 2q34-44, the entire chromosome (ch)3, 6q11-24, ch9, ch11, 12p11-q11, ch14, ch16, ch19, 5q35, and 6q32. There were also chromosomal losses on 1q23-26, 2q34-44, ch5, ch15, 15q12, and ch20 (Fig. 1a). Remarkable up-regulated and down-regulated genes in these chromosomes are summarized in Table 1. These findings suggest that the ESCC-DR cell line is genetically quite unstable. Among them, we selected ezrin, encoded by *vil2*, which was located in 1q11. Seven plots of ezrin in the CGH

**Table 1. Remarkably up-regulated and down-regulated genes detected using an Agilent rat 244K array in esophageal squamous cell carcinoma (ESCC)-DR cells**

| | |
|---|---|
| *(a) Up-regulated genes* | |
| Ch1 | AB191343, AY321318, AY462094, Ablim1-predicted, RGD1305481, Hivep2, Arhgap17, Arid1b, MGC94288, AY325187, Acat2, Dhhc14, Synj2, vil2, Fndc1 |
| Ch2 | M74152, Lphn2, Sv2c, RGD1309487, Slc1a3 |
| Ch4 | Ly49i9, Cftr, Reln |
| Ch5 | AY325175, Sulf1, LOC360689 |
| Ch6 | AJ286177, RGD1359539, BC091247, AJ286177, L07401, LOC366772, LOC364556 |
| Ch9 | Pthr2 |
| Ch11 | Impg2, Trp63, DQ394091, Dnm1l |
| Ch12 | Katnal1, MGC112692, Nudt1, EF535528, Flt1, G10, MGC125213, Sybl1, Cyp3a3, RGD1311893, RGD1307594, Zfp99, AF245000, Baiap2l1, Zdhhc4, MGC125213, Arpc1a, Cyp3a18, RGD1306873, Ocm, Kdelr2, Rac1, MGC116363, LOC498145, Zfp655, Rasl11a, Slc7a1, Pdap1, Alox5ap, RGD1306410, AY094358, RGD1311314, Nptx2, MGC112727, Baiap2l1, Kiaa0415, BC127538, BC129116, MGC125271, Azgp1, Sybl1, Alox5ap, RGD1311893, BC086602, Cyp3a11, Eif2ak1, LOC498155, MGC108785, Gna12, Eif3s9, G10, Cdx2, Ubl3, Bri3, RGD1311980, Taf6, Wipi2, Ap4m1, AY321325, LOC498152, Pscd3, Gpcr12, Bhlhb8, Zfp655, Pdap1, Zipro1, Actb, RGD1307594, EF535528 |
| Ch13 | C4bpa |
| Ch14 | Ugt2b, Slc4a4, Csnd |
| Ch16 | AF194993 |
| Ch18 | B4galt6 |
| Ch19 | Sipa1l2 |
| Ch20 | Pde9a |
| *(b) Down-regulated genes* | |
| Ch1 | Parva, Tmem23, AB113213, Cnnm2, RGD1309948, D37934, Txnl2, Pde10a, Slk, Tacc2, Grin2d, RGD1309578, Ppp2r2d |
| Ch2 | LOC360689, Tacr3, Rasgrf2, BC107651, AF536979, Rnf29, Ndfs4 |
| Ch3 | Arfgef2, Dnttip1, Tanc1, Pacsin3 |
| Ch4 | Grm7, M87784, DQ100481, S64522, C1rl, HK2, AF217592 |
| Ch5 | Nfia, AF112455, BCO088292, Ece1, Ppap2b, Mmp16, Dbccr1 |
| Ch6 | Z93359, Strn3, Myt1, Galnt14, Xdh, Tssc1 |
| Ch7 | Nell2, Hcfc2, AY321318 |
| Ch8 | AY383663, Hnt, Camkv |
| Ch9 | Dlgap1, Bard1 |
| CH10 | Grin2a, LOC498973, Cacng4 Masp1, Kcnj6, BC088235, Pou1f1 |
| Ch11 | Masp1, Kcnj6, BC088235, Pou1f1 |
| Ch12 | Cdv1 |
| Ch13 | Niban, Pla2g4a |
| Ch14 | AY321318, LOC498400, U83119 |
| Ch15 | BC088274, LOC290071, AF196255, AY321318, AF196255, DQ100479, Kcnma1, Tdrd3, AF196255, BC088274, LOC305691, Lect1, Spetex-2H, Cadps, Tdrd3, Acox2 |
| Ch16 | Dlgap2, Nrg1, Fgl1 |
| Ch17 | Srd5a1 |
| Ch18 | Sil1 |
| Ch19 | Got2, Ces3 |
| Ch20 | Btbd9, Tmlhe, DQ100478, AY383688 |

The cut-off value was as follows: 2.0 of $\log_2$ ratios for up-regulated genes and −2.0 for down-regulated genes.


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array are shown in Figure 1(b). The average of all plots was 1.917 ± 0.203 (average ± SD) compared to normal control esophageal epithelium.

**Ezrin and pERM protein levels in ESCC-DR cells and normal esophageal epithelium.** We performed a Western blot to identify ezrin expression in normal lung tissue, ESCC-DR cells, and normal esophageal epithelium. Ezrin and pERM expression were up-regulated in the ESCC-DR cells compared to normal esophageal epithelium (Fig. 2).

**Western blotting of ezrin and pERM obtained from fractionated ESCC-DR cells.** ESCC-DR cells were fractionated by differential centrifugation into P1 (nuclei), P2 (mitochondria and lysosomes), P3 (microsomal and plasma membrane), and S3 (cytosol) fractions, and subjected to Western blotting using anti-Ezrin or anti-pERM antibodies. Ezrin expression was detected in P1, P2, P3, and S3 fractions, whereas pERM expression was predominant in P3, but was also weakly detected in S3 and P2 (Fig. 3a).

**Ezrin and pERM expression in ESCC-DR cells.** Ezrin stained primarily in the cytoplasm and cell membranes (Fig. 3b). However, nuclei of several cells expressed ezrin. pERM was expressed mainly in the cell membranes, and weak cytoplasmic staining was also noted (Fig. 3c).

**Hematoxylin–eosin staining and immunohistochemistry of ezrin in various lesions.** Overall, the immunohistochemical staining patterns were broadly similar for each lesion. Ezrin distribution was different among non-neoplastic lesions, benign lesions, and ESCCs. Ezrin immunoreactivity was apparent in the cell membranes and weakly positive in the cytoplasm in normal esophageal mucosa and hyperplastic samples (Fig. 4a,b). In contrast, dysplastic and ESCC samples showed both membranous and cytoplasmic ezrin staining, and cytoplasmic staining increased in the these samples compared to the normal esophageal mucosa and hyperplastic samples (Fig. 4c,d). Several atypical cells with nuclear ezrin staining were also noted in ESCC samples. These findings suggested that ezrin was amplified in the carcinogenesis step of ESCCs, but nuclear expression of ezrin was detected only in cancerous lesions.

pERM immunoreactivity was weakly positive mainly in the cell membranes in normal esophageal mucosa and hyperplastic samples (Fig. 5a,b). Dysplastic and ESCC samples also showed

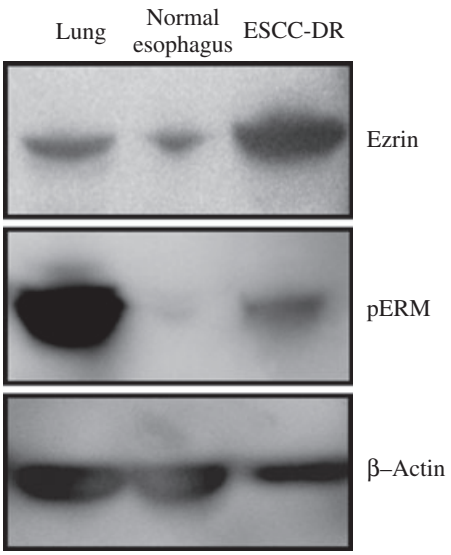


**Fig. 2.** Western blots of ezrin and pERM (phosphorylated ezrin, radixin, moesin) in esophageal squamous cell carcinoma (ESCC)-DR cells. β-Actin expression served as an internal control for equal loading. Normal lung was a positive control for ezrin and pERM. Ezrin and pERM were overexpressed in ESCC-DR cells compared to normal esophageal epithelial tissues. However, the pERM expression level was not as high in ESCC-DR cells compared to that of normal lung as a positive control.

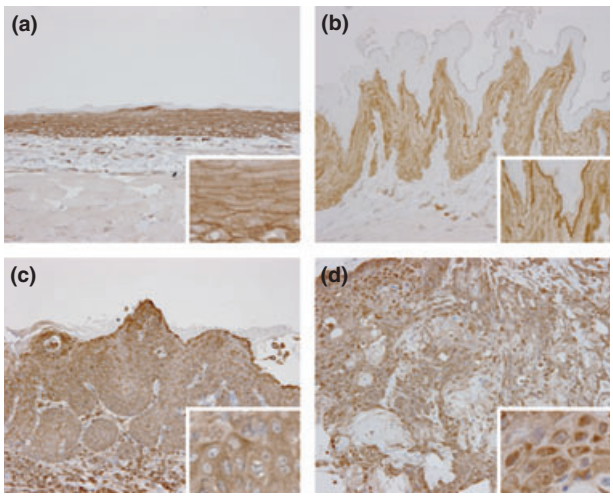


**Fig. 4.** Immunohistochemical staining of ezrin expression in various lesions (a–d: original magnification, ×200). Insets in each photo are a higher magnification of each lesion. Ezrin immunoreactivity was apparent in the membrane in normal esophageal epithelium (a) and in hyperplastic lesions (b), whereas positive staining for ezrin was mainly in the cytoplasm or in the membrane and cytoplasm of dysplastic and esophageal squamous cell carcinoma (ESCC) cells. The staining in neoplastic cells was much up-regulated (c,d). Moreover, ezrin immunoreactivity was focally apparent in the nucleus in ESCC (d).

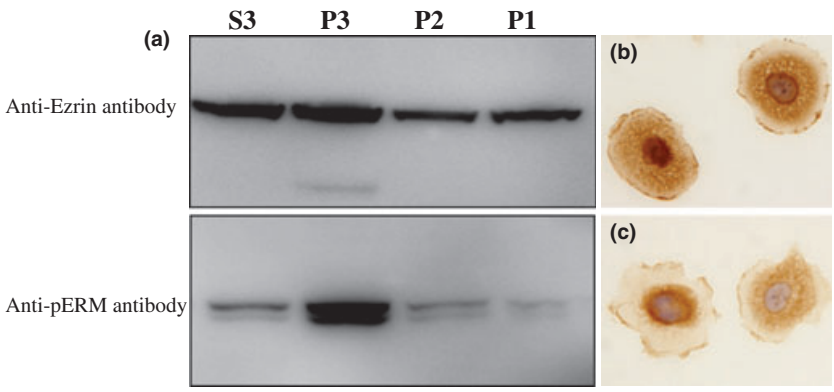


**Fig. 3.** Western blot and immunohistochemical staining of ezrin and pERM (phosphorylated ezrin, radixin, moesin) in fractionated esophageal squamous cell carcinoma (ESCC)-DR cells. ESCC-DR cells were fractionated by differential centrifugation into P1 (800$g$ pellet), P2 (10 000$g$ pellet), P3 (100 000$g$ pellet), and S3 (100 000$g$ supernatant) fractions (a). Ezrin expression was detected in P1, P2, P3, and S3 fractions, whereas pERM expression was predominant in P3 (a). Ezrin stained primarily in the cytoplasm and cell membranes. Nuclei of several cells expressed ezrin (b). pERM was expressed mainly in cell membranes, and cytoplasmic staining was also noted (c).

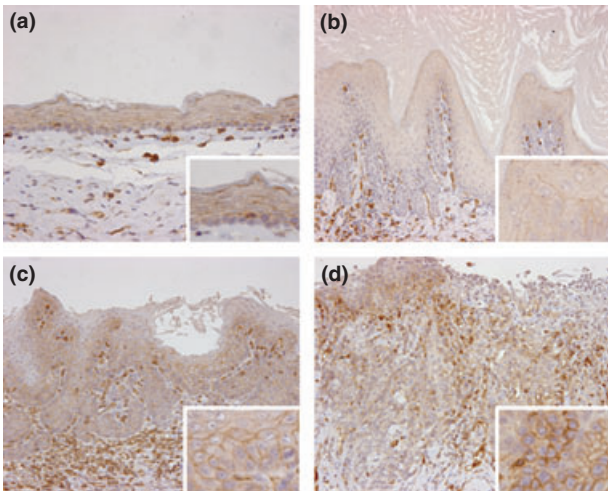


**Fig. 5.** Immunohistochemical staining of pERM (phosphorylated ezrin, radixin, moesin) expression in various lesions (a–d: original magnification, ×200). Insets in each photo are a higher magnification of each lesion. pERM immunoreactivity was weakly positive mainly in the cell membranes in normal esophageal mucosa and hyperplastic samples (a,b). Dysplastic and esophageal squamous cell carcinoma (ESCC) samples showed strongly positive staining in the membrane (c,d).

mainly membranous staining, and the membranous staining increased compared to the normal esophageal mucosa and hyperplastic samples (Fig. 5c,d). However, pERM was expressed only at the invasive front in ESCC samples.

In ESCC cells detected in lymphatic vessels of the lungs (Fig. 6a), ezrin expression was distributed mainly in the cytoplasm (Fig. 6b), whereas pERM expression was detected weakly in the cytoplasm and cell membrane of several ESCC cells (Fig. 6c). Small metastatic foci in the lungs expressed ezrin uniformly in the cytoplasm (Fig. 6d,e); however, pERM was not expressed (Fig. 6f). In larger metastatic lesions that progressed to the lymph nodes and lungs (Fig. 7a,e), ezrin expression was uniformly high and distributed mainly in the cytoplasm and partly in the cell membrane (Fig. 7b,f), and pERM was re-expressed, but only at the periphery of the expanding metastatic lesions (Fig. 7c,d,g,h).

## Discussion

It is well known that ESCC is associated with smoking and alcohol consumption.[3–5] On the other hand, several reports have indicated that tobacco and alcohol abuse decrease lower esophageal motility, delay gastric emptying, and increase gastric secretion.[36–40] These studies have thus demonstrated the refluxogenic effects of smoking and alcohol abuse, and support a putative role for gastroduodenal content reflux as a factor associated with the development of ESCC. Although it has been widely accepted that duodenogastric-esophageal reflux is directly linked to Barrett's esophagus and the development of EAC and no risk for ESCC has been associated with gastroesophageal reflux, results of several studies using rat duodenal content reflux models have shown the development of not only EAC but also ESCC.[7–9,41] Thus, duodenal content-reflux probably plays an important role on developing ESCC as well as EAC. It is unclear what factors lead to the formation of carcinomas of specified histological types. Miwa *et al.* suggested that ESCC develops in places distant from the anastomosis as compared to EAC. This finding means that histological features may depend upon the volume of reflux contents; continuous but minimal amounts of reflux causes ESCC, and a large volume of reflux causes EAC.[41]

In a duodenal content-reflux model of rats that did not involve the use of any known carcinogens, we reported ESCCs with thoracic dissemination and metastases in the lymph nodes that developed at 60 weeks' postoperation and established an ESCC-DR cell line from the metastatic tumor.[9] In the present study, many chromosomal losses and gains were detected by a CGH array analysis in the ESCC-DR cells. These facts indicate that duodenal reflux can induce genetically unstable as well as highly malignant tumors. These results have confirmed that duodenal reflux can initiate malignancy. We previously evaluated the inhibitory effects of thiazolidine-4-carboxylic acid (thioproline) as a nitrite scavenger and a highly sensitive probe to detect *N*-nitroso compounds using duodenal content-reflux animals and found that thioproline prevented esophageal and remnant stomach carcinogenesis, thereby indicating the possible role of *N*-nitroso compounds in carcinogenesis.[7,42] Moreover, we recently reported that *N*-nitrosotaurocholic acid and *N*-nitrosoglycocholic acid are formed by nitrosation of taurocholic acid and glycocholic acid, respectively. These nitroso compounds produce DNA adducts in the glandular stomach of rats subjected to duodenal content reflux surgery.[43] These findings also suggest that duodenal reflux can potentially initiate malignancy, and the driving force of carcinogenesis is probably *N*-nitroso bile acids formed in the upper digestive tract *in vivo*.

Of the many genes identified in the present CGH array, we detected an interesting ezrin amplicon, which has been recently reported in human ESCC. As a member of the ERM protein family, ezrin acts both as a linker between the actin cytoskeleton and plasma membrane proteins and as a signal transducer in

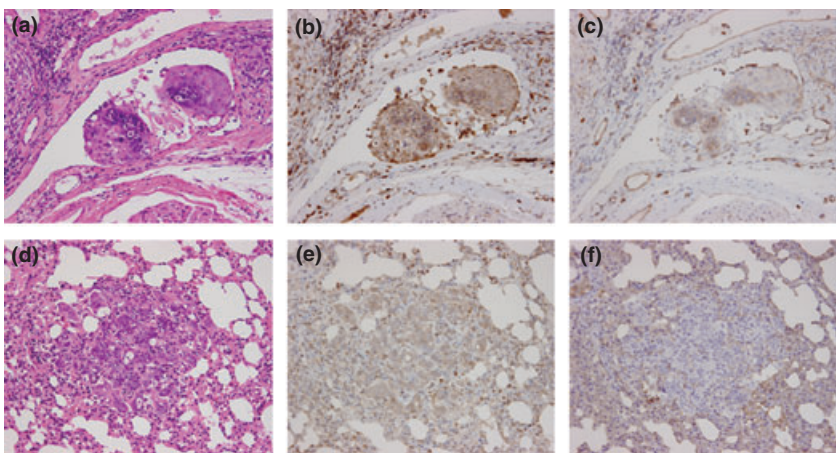


**Fig. 6.** Immunohistochemistry of ezrin and pERM (phosphorylated ezrin, radixin, moesin) in lymphatic involvement (a–c) and small metastatic foci in lung (d–f) (a–f: original magnification, ×200). (a,d) hematoxylin–eosin (HE), (b,e) ezrin, (c,f) pERM. In the atypical esophageal squamous cell carcinoma (ESCC) cells in the lymph duct of the lung (a), ezrin expression was uniformly high (b), where pERM expression was partly detected (c). Small metastatic foci expressed ezrin uniformly (d,e); however, pERM was not expressed (f).


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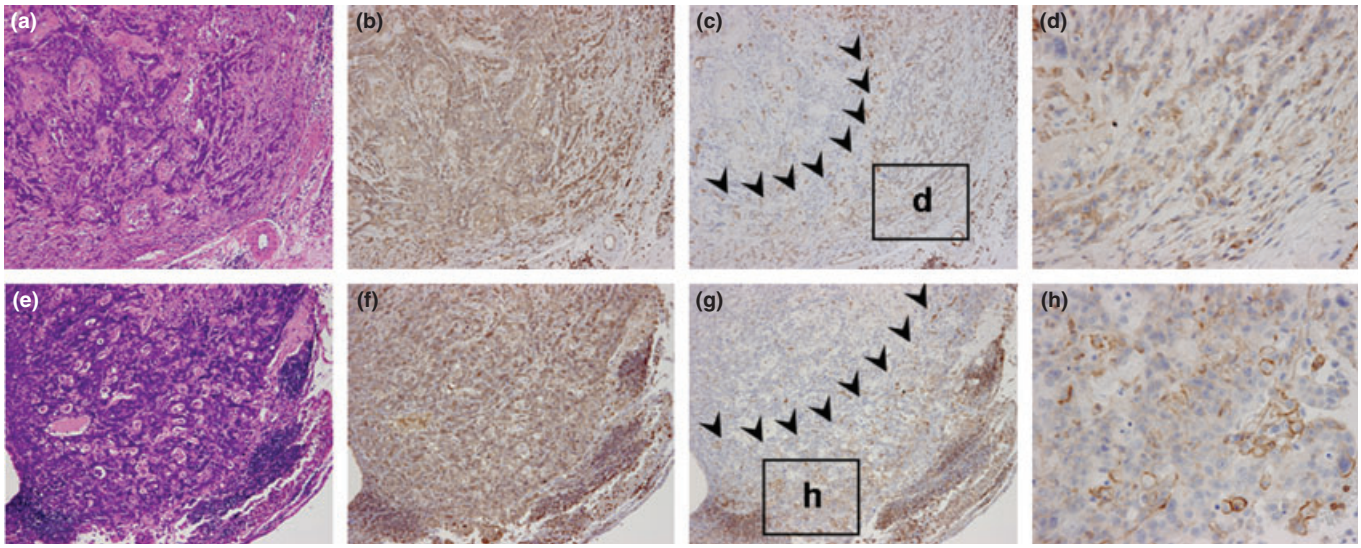


**Fig. 7.** Immunohistochemistry of ezrin and pERM (phosphorylated ezrin, radixin, moesin) in larger metastatic sites: in lymph nodes (a–d) and in the lung (e–h) (a–c and e–g: original magnification, ×100; d,h: original magnification, ×400). (a,e) HE, (b,f) ezrin (c,d,g,h) pERM. Ezrin expression was uniformly high and distributed mainly in the cytoplasm (b,f), and pERM was re-expressed but only at the periphery of the expanding metastatic lesions (c,d,g,h).

responses involving cytoskeletal remodelling,[12] and its overexpression is required in some tumor tissues or cell lines for metastasis.[30] Some work has already been done on the roles of ezrin in human ESCC, and up-regulated ezrin expression is probably associated with the invasive phenotype of malignantly transformed esophageal epithelial cells.[44] Moreover, recent literature on esophageal epithelial tissues showed that ezrin has a tendency to translocate from the plasma membrane to the cytoplasm during the progression from a normal epithelium to an invasive carcinoma of the esophagus.[11] In addition, it has been reported that nuclear expression of ezrin may be also involved in the progression of ESCC.[10]

Few studies have confirmed that ezrin is a unique ERM protein involved in the metastatic process. In some cases, the contribution of ezrin to the metastatic phenotype may be exploited once activated, by providing a physical connection between the plasma membrane and actin cytoskeleton. In metastatic melanoma, ezrin mediates the aberrant linkage of the cytoskeleton to various proteins, including CD44 and LAMP-1, inducing marked changes in the general framework of cellular function. This also leads to an aberrant engagement with the extracellular microenvironment, that is directly involved in metastatic behavior of tumor cells.[28,45] Ezrin can exist in an active or dormant state. The active form is involved in interactions with membrane molecules, whereas the dormant form is present in the cytoplasm.[46] Thus, we can deduce that ezrin may exist predominantly in an active state in normal esophageal mucosal epithelium, whereas concomitant actin cytoskeleton breaks down in ESCC cells, which are mainly in a dormant state. In the present study, we showed that ezrin is not only constitutively found in membranes but also in the cytoplasm and focally in the nucleus of ESCC cells. These findings are similar to those reported in human cases of ESCC.[11] Recent literature has reported that ERM is phosphorylated early after metastatic cells arrive in the lung. As these multicellular lesions progress, they lose pERM expression. However, as these lesions grow even larger, pERM is expressed again, but only at the leading edge, or invasive front, of the lesions.[47] We also examined pERM expression using ESCC samples and metastatic sites in lymph nodes and lung developed in duodenal content-reflux animals. The pattern of expression observed in the present study was quite similar to a previous study using osteoblastoma cell lines .[47] We could not check soon after metastatic cells arrived in the lung, but we could examine ezrin and pERM expression in atypical ESCC cells of the lymph duct and small metastatic foci in the lungs. We also studied larger metastatic sites in the lymph nodes and lungs. We detected pERM expression in the circulating atypical cells, but not in small metastatic foci. These data suggest that phosphorylation is only required in metastatic behavior of tumor cells. pERM may play a role in an aberrant engagement with the extracellular microenvironment, which is directly involved in the metastatic behavior of tumor cells at the leading edge and invasive front.

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