References

1. Ghadimi BM, Sackett DL, Difilippantonio MJ, et al. Centrosome amplification and instability occurs exclusively in aneuploid, but not in diploid colorectal cancer cell lines, and correlates with numerical chromosomal aberrations. Genes Chromosomes Cancer 2000;27:183–90.
2. Kuo KK, Sato N, Mizumoto K, et al. Centrosome abnormalities in human carcinomas of the gallbladder and intrahepatic and extrahepatic bile ducts. Hepatology 2000;31:59–64.
3. Lingle WL, Lutz WH, Ingle JN, Maihle NJ, Salisbury JL. Centrosome hypertrophy in human breast tumors: implications for genomic stability and cell polarity. Proc Natl Acad Sci U S A 1998;95:2950–5.
4. Pihan GA, Purohit A, Wallace J, Malhotra R, Liotta L, Doxsey SJ. Centrosome defects can account for cellular and genetic changes that characterize prostate cancer progression. Cancer Res 2001;61:2212–9.
5. Pihan GA, Purohit A, Wallace J, et al. Centrosome defects and genetic instability in malignant tumors. Cancer Res 1998;58:3974–85.
6. Sato N, Mizumoto K, Nakamura M, et al. Correlation between centrosome abnormalities and chromosomal instability in human pancreatic cancer cells. Cancer Genet Cytogenet 2001;126:13–9.
7. Saunders WS, Shuster M, Huang X, et al. Chromosomal instability and cytoskeletal defects in oral cancer cells. Proc Natl Acad Sci U S A 2000;97:303–8.
8. Zhou H, Kuang J, Zhong L, et al. Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy, and transformation. Nat Genet 1998;20:189–93.
9. Bischoff JR, Anderson L, Zhu Y, et al. A homologue of *Drosophila* aurora kinase is oncogenic and amplified in human colorectal cancers. EMBO J 1998;17:3052–65.
10. Li D, Zhu J, Firozi PF, et al. Overexpression of oncogenic STK15/BTAK/Aurora A kinase in human pancreatic cancer. Clin Cancer Res 2003;9:991–7.
11. Sen S, Zhou H, Zhang RD, et al. Amplification/overexpression of a mitotic kinase gene in human bladder cancer. J Natl Cancer Inst 2002;94:1320–9.
12. Royce ME, Xia W, Sahin AA, et al. STK15/Aurora-A expression in primary breast tumors is correlated with nuclear grade but not with prognosis. Cancer 2004;100:12–9.
13. Hamada M, Yakushijin Y, Ohtsuka M, Kakimoto M, Yasukawa M, Fujita S. Aurora2/BTAK/STK15 is involved in cell cycle checkpoint and cell survival of aggressive non-Hodgkin's lymphoma. Br J Haematol 2003;121:439–47.
14. Tanaka E, Hashimoto Y, Ito T, et al. The clinical significance of Aurora-A/STK15/BTAK expression in human esophageal squamous cell carcinoma. Clin Cancer Res 2005;11:1827–34.
15. Anand S, Penrhyn-Lowe S, Venkitaraman AR. AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol. Cancer Cell 2003;3:51–62.
16. Dutertre S, Prigent C. Aurora-A overexpression leads to override of the microtubule-kinetochore attachment checkpoint. Mol Interv 2003;3:127–30.
17. Hata T, Furukawa T, Sunamura M, et al. RNA interference targeting aurora kinase a suppresses tumor growth and enhances the taxane chemosensitivity in human pancreatic cancer cells. Cancer Res 2005;65:2899–905.
18. Isono K, Sato H, Nakayama K. Results of a nationwide study on the three field lymph node dissection of esophageal cancer. Oncology 1991;48:411–20.
19. Pisani P, Parkin DM, Bray F, Ferlay J. Estimates of the worldwide mortality from 25 cancers in 1990. Int J Cancer 1999;83:18–29.
20. Muro K, Hamaguchi T, Ohtsu A, et al. A phase II study of single-agent docetaxel in patients with metastatic esophageal cancer. Ann Oncol 2004;15:955–9.
21. Lordick F, von Schilling C, Bernhard H, Hennig M, Bredenkamp R, Peschel C. Phase II trial of irinotecan plus docetaxel in cisplatin-pretreated relapsed or refractory oesophageal cancer. Br J Cancer 2003;89:630–3.
22. Pimkhaokham A, Shimada Y, Fukuda Y, et al. Nonrandom chromosomal imbalances in esophageal squamous cell carcinoma cell lines: possible involvement of the ATF3 and CENPF genes in the 1q32 amplicon. Jpn J Cancer Res 2000;91:1126–33.
23. Shimada Y, Imamura M, Wagata T, Yamaguchi N, Tobe T. Characterization of 21 newly established esophageal cancer cell lines. Cancer 1992;69:277–84.
24. Cremet JY, Descamps S, Verite F, Martin A, Prigent C. Preparation and characterization of a human aurora-A kinase monoclonal antibody. Mol Cell Biochem 2003;243:123–31.
25. Marumoto T, Honda S, Hara T, et al. Aurora-A kinase maintains the fidelity of early and late mitotic events in HeLa cells. J Biol Chem 2003;278:51786–95.
26. Khvorova A, Reynolds A, Jayasena SD. Functional siRNAs and miRNAs exhibit strand bias. Cell 2003;115:209–16.
27. Dandekar DS, Lokeshwar BL. Inhibition of cyclooxygenase (COX)-2 expression by Tet-inducible COX-2 antisense cDNA in hormone-refractory prostate cancer significantly slows tumor growth and improves efficacy of chemotherapeutic drugs. Clin Cancer Res 2004;10:8037–47.
28. Dutertre S, Cazales M, Quaranta M, et al. Phosphorylation of CDC25B by Aurora-A at the centrosome contributes to the G_2-M transition. J Cell Sci 2004;117:2523–31.
29. Brummelkamp TR, Bernards R, Agami R. Stable suppression of tumorigenicity by virus-mediated RNA interference. Cancer Cell 2002;2:243–7.
30. Hingorani SR, Jacobetz MA, Robertson GP, Herlyn M, Tuveson DA. Suppression of BRAF(V599E) in human melanoma abrogates transformation. Cancer Res 2003;63:5198–202.
31. Takei Y, Kadomatsu K, Yuzawa Y, Matsuo S, Muramatsu T. A small interfering RNA targeting vascular endothelial growth factor as cancer therapeutics. Cancer Res 2004;64:3365–70.
32. Mortlock AA, Keen NJ, Jung FH, et al. Progress in the development of selective inhibitors of aurora kinases. Curr Top Med Chem 2005;5:807–21.
33. Keen N, Taylor S. Aurora-kinase inhibitors as anticancer agents. Nat Rev Cancer 2004;4:927–36.

Esophagus (2007) 4:7–18
DOI 10.1007/s10388-006-0101-9

REVIEW ARTICLE

Yutaka Shimada · Fumiaki Sato

Molecular factors related to metastasis of esophageal squamous cell carcinoma

Received: December 22, 2006

Abstract The aggressive behavior of esophageal cancer leads to a low survival rate for patients with this disease. Isolated esophageal cancer cells seem to have the potential for regrowth and metastasis. To control the metastasis of esophageal carcinoma, detailed analysis of various molecular and biological factors should be done in each patient. Recent progress in molecular biology has revealed that oncogenes, suppressor genes, growth factors and their receptors, adhesion molecules, angiogenic factors, cytokines, and apoptotic factors are related to the growth and metastasis of esophageal carcinoma. Among them, several genes have been identified as having a crucial role in establishing the metastasis of esophageal carcinoma, and multiple genetic alterations may underlie the metastatic process. Microarray analysis has also revealed several genetic networks that are involved in the metastasis of esophageal cancer. These recent findings regarding the metastasis of esophageal cancer are summarized in the present review.

Key words Esophageal cancer · Lymph node metastasis · Hematogenous metastasis · Isolated tumor cell · Metastatic potential

Introduction

Esophageal cancer is the sixth most frequent cause of cancer death worldwide, which is a higher rank than its incidence (eighth) because of the low survival rate [1,2]. According to the Surveillance Epidemiology and End Results (SEER) program, the stage distribution of patients with esophageal cancer diagnosed at the U.S. National Cancer Institute was as follows: one-third of the patients had localized disease (no sign of progression), one-third of them had regional disease (usually with lymph node involvement), and the remaining one-third had distant metastasis [3]. Thus, understanding the mechanism of metastasis for esophageal cancer may help us to treat this deadly disease more effectively.

There are several steps in the process of establishment of metastasis by cancer [3,4]. The initial phase is an invasive process and the second phase is a metastatic process. The invasive process has several steps, which are (1) loss of contact inhibition, (2) tumor vascularization, (3) detachment of cells from the primary tumor, (4) penetration of the basement membrane, and (5) migration through the stroma. The metastatic process also has several steps, comprising (1) penetration of lymphatics and/vessels, (2) escape from immune surveillance and survival in the circulation, (3) entrapment in the capillary bed of a distant organ, (4) penetration of the lymphatic or vessel wall to enter the tissue, (5) growth in the new location, and (6) tumor vascularization. Thus, invasion and metastasis are both dynamic multistep processes [4].

With continued advances in the field of molecular biology, our understanding of the behavior of esophageal cancer continues to evolve. The available evidence suggests that multiple genetic alterations at the nucleotide and chromosomal levels may be involved in the metastasis of esophageal cancer. These changes may include alterations of oncogene or tumor suppressor genes, as well as genes for growth factors, angiogenic factors, adhesion molecules, and cytokines, and those involved in signal transduction and apoptosis. Recent findings regarding the metastasis of esophageal cancer are summarized in this review.

Invasion

Invasion initiates the metastatic process and requires changes of tumor cell adhesion to other cells and to the extracel-

Y. Shimada (✉)
First Department of Surgery, Hyogo College of Medicine,
1-1 Mukogawacho, Nishinomiya, Hyogo 663-8501, Japan
Tel. +81-798-45-6582; Fax +81-798-45-6581
e-mail: sshimada@hyo-med.ac.jp

F. Sato
Division of Gastroenterology and Hepatology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA

lular matrix (ECM), as well as proteolytic degradation of the tissue matrix and increased motility of tumor cells. Then, it is necessary for tumor cells to penetrate into the lymphatics or blood vessels.

Cadherins

Cell-to-cell adhesion is mediated by cadherins, and E-cadherin is the main regulator in epithelial tissues [5]. Loss or dysfunction of E-cadherin decreases intercellular adhesion and results in the acquisition of invasive capacity by esophageal cancer cell lines [6]. Epidermal growth factor (EGF) counteracts E-cadherin-mediated junctional assembly through phosphorylation of beta-catenin and allows tumor cell behavior to become more aggressive [7]. Also, hematogenous recurrence of esophageal squamous cell carcinoma (ESCC) is reported to show a correlation with E-cadherin expression [8].

Alpha-catenin (alpha-cat) is a cytoplasmic protein that forms a link to the cytoskeleton and thus regulates the activity of E-cadherin (E-cad) [9]. The frequency of lymph node metastasis by E-cad(+/–)/alpha-cat(–) tumors is significantly higher than by E-cad(+)/alpha-cat(+) tumors or E-cad(+/–)/alpha-cat(+/–) tumors. Thus, not only E-cad but also alpha-cat are important regulators of intercellular adhesion. In particular, reduction of alpha-cat expression in human esophageal cancer is more strongly correlated with an invasive phenotype and lymph node metastasis than increased E-cad expression [10].

Although such data exist, two multicenter studies have failed to confirm any relation between E-cadherin expression and lymph node metastasis or hematogenous metastasis in patients with either advanced or superficial esophageal cancer [11,12]. Thus, other mechanisms are suspected to contribute to cadherin-related regulation of cell-to-cell attachment in patients with esophageal cancer. The *Snail* gene product is a transcriptional repressor of E-cadherin and an inducer of the epithelial-mesenchymal transition in several epithelial tumor cell lines [13,14]. Deletion of E-cadherin is regulated by hypermethylation and Snail expression, but overexpression of Snail is unrelated to clinicopathological factors [15]. On the other hand, *Slug*, a member of the *Snail* family of transcriptional factors, is a newly identified suppressor of E-cadherin transcription [16]. Slug expression is significantly correlated with reduced E-cadherin expression, and tumors with low E-cadherin expression or positive Slug expression are more likely to show lymph node metastasis than tumors with preserved E-cadherin expression or negative Slug expression [17].

Adhesion molecules

TSLC1/IGSF4 has been identified on chromosome 11q23.2 as a tumor suppressor gene in non-small cell lung cancer by functional complementation of a lung adenocarcinoma cell line [18]. In a clinicopathological study, loss of TSLC1 expression by ESCC was significantly correlated with the depth of invasion and the metastatic status. The in vivo motility and invasiveness of ESCC cells were significantly suppressed by TSLC1 transfection in vivo [19].

Thrombomodulin (TM) is a thrombin receptor that was originally identified on the endothelium and acts as a natural anticoagulant [20]. However, TM is also expressed by squamous epithelial cells, mainly at intercellular bridges. Tumors usually express TM at cell–cell boundaries and in the cytoplasm, and a decrease of TM expression is associated with lymph node metastasis. This action is very similar to the influence of E-cadherin, although the structure of the two molecules is quite different [21].

Desmoglein 1 (DG1) is a major component of the desmosomal membrane core that plays an important role in epithelial cell adhesion [22]. A strong inverse correlation has been found between DG1 expression and tumor invasion, lymph node metastasis, lymphatic invasion, and vascular invasion [23].

Focal adhesion kinase (FAK) is a tyrosine kinase that is localized to focal cellular adhesions and is associated with a number of other proteins, such as integrin adhesion receptors [24]. A significant correlation has been observed between FAK overexpression and the presence of regional lymph node metastasis, as well as the number of involved lymph nodes [25].

Thus, not only the cadherin cell–cell adhesion system, but also various other adhesion molecules, including TSLC1, thrombomodulin, desmoglein 1, and FAK, play a role in the metastasis of esophageal squamous cell carcinoma.

Matrix metalloproteinases

Degradation of the extracellular matrix (ECM) is mediated by matrix metalloproteinases (MMPs), and this is one of the key steps during tumor invasion and metastasis [26]. MMPs are known to be involved in the cleavage of cell-surface receptors, the release of apoptotic ligands, and inactivation of chemokines. MMPs are also thought to play a major role in various aspects of cell behavior, such as proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis, and host defenses.

Among the many MMPs, the expression of MMP-2, -3, -7, -9, -12, -13, -14, and -26 appears to be closely related to lymph node metastasis of ESCC [27–32]. In addition, MMP-26 (matrilysin-2) expression is significantly correlated with distant metastasis [32].

Tissue inhibitors of metalloproteinases (TIMPs) inhibit the activity of MMPs and may also play an important role in tumor invasion/metastasis [26]. TIMP-1 and -3 have been associated with lymph node metastasis of ESCC [33,34].

Growth potential and motility

Cell proliferation

Cyclin D1 has been mapped to the 11q13 region of chromosome 11, and its expression has been detected in SCC cell lines and several primary esophageal cancers [35,36]. Overexpression of *cyclin D1* is correlated with distant metastasis after curative resection, but there is no significant difference in lymph node recurrence between patients with or without overexpression [37,38]. This relationship of cyclin D1 to hematogenous metastasis has been confirmed by logistic regression analysis based on 11 different molecular and cellular factors [39]. However, the reason why cyclin D1 is related to hematogenous metastasis, but not to lymph node metastasis, is unclear.

The *p16/MTS1/CDKN2* gene regulates cyclin-dependent kinase 4-mediated phosphorylation of pRB in normal cells. The mean number of metastatic lymph nodes was significantly higher in patients who had ESCC without p16 expression than in patients whose tumors showed p16 expression [38].

Ornithine decarboxylase (ODC) is a key enzyme involved in the biosynthesis of polyamines, which are essential for cell proliferation [40]. ODC-overproducing transfectants show enhanced MAP kinase activity that parallels the extent of their invasiveness [41]. In patients with esophageal cancer, overexpression of ODC mRNA shows a significant correlation with lymph node metastasis [42].

Aurora-A/STK15/BTAK (Aurora-A) encodes a serine/threonine kinase associated with the distribution of chromosomes, and its upregulation induces chromosomal instability that leads to aneuploidy and cell transformation [43]. A correlation between the upregulation of Aurora A and tumor aggressiveness has also been detected for several cancers [44], and upregulation of Aurora-A mRNA and protein is correlated with distant lymph node metastasis of ESCC [45].

Thus, various genes related to cell proliferation may have a role in the metastasis of esophageal cancer.

Motility

Growth factors

Epidermal growth factor (EGF) is one of the best known growth factors expressed by malignant tumors, and upregulation of the *EGF receptor (EGFR)* gene is associated with a poor prognosis of ESCC [46,47]. Although the EGFR is thought to have a role in metastasis of ESCC, there has only been one study that demonstrated an association between lymph node metastasis of ESCC and EGFR expression [48].

Nerve growth factor (NGF) is overexpressed not only in the nervous system but also by several types of cancer [49,50]. An immunohistochemical study has revealed that NGF overexpression is associated with lymph node metastasis and distant metastasis of ESCC [51]. NGF overexpression is also associated with strong TrkA expression and with the lack of low-affinity neurotrophin receptor (p75NTR) expression. Cultured ESCC cell lines secrete detectable amounts of NGF and also express both TrkA and p75NTR. A neutralizing anti-NGF antibody, a TrkA inhibitor, and a small interfering RNA for NGF have all been shown to significantly decrease the motility of an ESCC cell line overexpressing NGF [51].

Signal transduction

Growth factor receptor-bound protein 7 (Grb7) has an Src homology 2 (SH2) domain and shares structural homology with a molecule related to cell migration in *Caenorhabditis elegans* (designated as Mig-10) [52,53]. In esophageal carcinoma cells, Grb7 protein undergoes tyrosine phosphorylation by epidermal growth factor and becomes attached to various extracellular matrix proteins, including fibronectin. This fibronectin-dependent phosphorylation of Grb7 is regulated by integrin signaling that leads to an interaction with focal adhesion kinase protein. Ectopic expression of a Grb7-SH2 dominant-negative fragment has been found to inhibit fibronectin-dependent phosphorylation of endogenous Grb7 and reduce the migration of esophageal carcinoma cells. Grb7 protein overexpression in resected ESCC tumors is significantly correlated with the presence of lymph node metastasis [54].

Members of the *Smad* family play a key role in regulating gene expression in the transforming growth factor (TGF)-beta-1 signaling pathway [55]. Activation of Smads leads to their translocation from the cytoplasm to the nucleus, where these molecules act as transcription factors. Esophageal cancer patients with preserved expression of Smad4 have fewer lymph node metastases than those with reduced Smad4 expression. However, tumor cell expression of TGF-beta-1 is not associated with any clinicopathological factors [56].

Activin A is a member of the transforming growth factor-beta (TGF-beta) superfamily and is a strong differentiation factor for embryonic stem (ES) cells [57]. *Activin-betaA (Act-betaA)* is a subunit of activin A that shows significant overexpression in cancer tissue. Although expression of Act-beta A mRNA is not associated with the differentiation of ESCC, it is associated with lymph node metastasis [58].

The Ras homologue (Rho) family of small guanosine triphosphatases (GTPases) comprise a large branch within the Ras family of low molecular weight guanine nucleotide-binding proteins. Rho GTPases are thought to be involved in regulating diverse physiological responses, such as cell proliferation and motility, as well as pathological processes such as cell transformation and tumor metastasis [59]. Despite the marked similarity of the different Rho isoforms (Rho A, Rho B, and Rho C), their physiological roles are distinct. In transfected ESCC cells, transfection of RhoC promoted migration more effectively compared to RhoA [60]. In a nude mouse experimental metastasis model,

RhoA was shown to stimulate tumor growth more than RhoC, whereas RhoC induced lung metastasis more often than RhoA [60].

Recent studies have demonstrated that the Sonic Hedgehog (SHh) signaling pathway plays an important role in the development of various organs, including the neural tube, lungs, gastrointestinal tract, pancreas, and prostate [61]. SHh proteins act via the transmembrane proteins Patched (Ptch) and Smoothened (Smo), which regulate the transcriptional activity of three Gli zinc-finger transcription factors [61,62]. Increased Hh signaling can lead to the development of tumors (including gastrointestinal cancer), and activation of the Hh signaling pathway is associated with tumor metastasis [63,64].

Gli-1 was expressed by 31 of 34 esophageal cancer cell lines (91%), whereas SHh, Ptch, and Smo were expressed by all 34 cell lines [65]. Cyclopamine (a selective inhibitor of the Hh signaling pathway) significantly inhibited the proliferation and migration of ESCC cells expressing Gli-1. Also, SiRNA targeting Gli-1 inhibited the growth of ESCC cells. Furthermore, the detection of Gli-1 expression in resected tumors was associated with deeper tumor invasion, lymph node metastasis, and a poor prognosis [65].

Migration factors

Caveolin-1 is the principal structural component of the caveolar membrane domain in nonmuscle cells, including the ductal epithelium of the breast. There is now clear evidence that caveolin-1 influences the development of cancer in humans [66]. In cultured cells, caveolin-1 is known to act as a negative regulator of the Rasp42/44 MAP kinase cascade and as a repressor of *cyclin D1* gene transcription, possibly explaining its in vitro suppressive effect on cell transformation. However, overexpression of caveolin-1 is associated with lymph node metastasis of ESCC and with a worse prognosis after surgery [67].

Fascin is an actin-binding protein that induces membrane protrusions and increases the motility of various transformed cells [68]. An immunohistochemical study has revealed that fascin expression is usually stronger in tumors compared with normal epithelium. The fascin-immunopositive rate was associated with the extent of tumor invasion and with lymph node metastasis in ESCC patients [69], and an in vitro study revealed that all the 33 ESCC cell lines tested were positive for fascin protein expression. One of the fascin-overexpressing cell lines showed a decrease of motility and invasiveness after downregulation of fascin expression by a vector-based small interfering RNA [69].

Autocrine motility factor (AMP) and its receptor (gp78) have been shown to play an important role in tumor cell migration, invasion, and metastasis [70]. In ESCC, gp78 expression shows a significant association with tumor size, infiltrative growth, depth of invasion, and lymph node metastasis [71].

Laminin-5 gamma-2 chain (LN-5 gamma-2) is an extracellular matrix protein that plays an important role in cell migration and tumor invasion [72]. LN-5 gamma-2 expression in the invasive edge of the tumor was correlated with the depth of invasion and lymph node metastasis [48].

Although the mechanism of action of the transmembrane superfamilies, motility-related protein-1 (MRP-1/CD9), and KAI1/CD82 is not well known, these molecules have been reported to suppress the metastasis of several cancers [73,74]. Expression of both MRP-1/CD9 and KAI1/CD82 is positive in the membranes of normal esophageal epithelial cells but is reduced or negative in the case of cancer cells. Decreased expression of MRP-1/CD9 and KAI/CD-82 is significantly correlated with lymph node metastasis of ESCC, but not with distant metastasis [75].

Thus, growth factors, signal transduction players, and migration-related factors also contribute to the metastasis of esophageal cancer.

Metastasis

Some tumor cells are able to escape from the host immune system and survive in the blood vessels or lymphatics until they become trapped in the capillary bed of a distant organ. Then, these tumor cells penetrate the lymphatic or vessel wall and enter the tissues, after which metastatic deposits grow in the new location along with neovascularization. To carry out these steps, tumor cells always employ the same system, which is part of the invasion process.

Migration to specific organs

Chemokines

Tumor cell migration and metastasis share many similarities with leukocyte migration, which is regulated by various chemokines and their receptors. Among them, CC chemokine receptor 7 (CCR7) plays a critical role in the migration of activated dendritic cells into regional lymph nodes [76]. CCR7 mRNA was detected in 9 of 20 ESCC cell lines. In vitro studies have demonstrated that CCL21 (the ligand of CCR7) significantly increases the migration of ESCC cell lines, with pseudopodia being induced by CCL21 stimulation. Furthermore, CCL21 markedly enhances the motility of esophageal carcinoma cell lines in a phagocytosis assay. An immunohistochemical study has shown that high CCR7 expression is significantly correlated with lymphatic involved by ESCC and subsequent lymph node metastasis [77].

Bone marrow-homing receptor CXCR4 has also been suggested to have a role in the metastasis of various cancers [76]. CXCR4 expression has a significant association with increased lymph node micrometastasis and with increased bone marrow micrometastasis. Thus, CXCR4 may have a role in early metastatic spread because its expression is associated with micrometastasis to both the lymph nodes and bone marrow [78].

Isolated tumor cells

Lymph node micrometastasis

Although lymph node micrometastasis is thought to be a poor prognostic factor for ESCC, the clinical impact is still controversial [79,80]. Scheunemann et al. showed that there is a tumorigenic potential of apparently tumor-free lymph nodes in esophageal cancer patients [81]. Thus, prediction of micrometastasis may be useful for deciding on esophageal cancer treatment. However, the expression of E-cadherin, cyclin D1, MMP 9, and vascular endothelial growth factor by primary ESCC does not predict lymph node micrometastasis [82].

Circulating tumor cells

To detect circulating esophageal tumor cells, immunostaining for carcinoembryonic antigen (CEA) mRNA and SCC mRNA is usually employed [83–85]. The overall incidence of recurrence and that of hematogenous recurrence are significantly higher in patients who are positive for CEA mRNA than in those who are negative [84]. Detection of SCCA mRNA in the peripheral blood on admission is correlated with the depth of tumor invasion and with venous invasion. The recurrence rate is correlated with the SCC mRNA status on admission and during manipulation of the tumor [85]. Thus, the existence of circulating tumor cells is a risk factor for metastasis. Furthermore, a higher colony formation rate in cultures of blood samples is associated with the number of circulating tumor cells (Fig. 1). The establishment of metastasis thus may be associated with the number of circulating tumor cells and the ability to survive in culture.

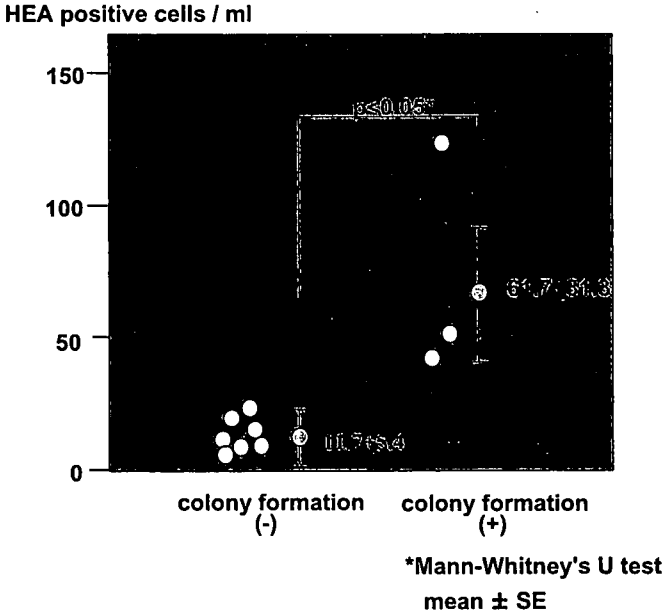

Fig. 1. Association between the number of isolated tumor cells (*ITC*) obtained with a magnetic cell sorter (*MACS*) and colony formation. A larger number of colonies in cultures from blood samples was associated with the number of circulating tumor cells. *HEA*, human epithelial antigen–125/EpCAM antigen

Free tumor cells in the thoracic duct

Watanabe et al. showed that thoracic duct fluid from ESCC patients had a high rate (61%: 8/13) of tumorigenicity in nude mice [86]. This very high rate of tumorigenicity suggests that esophageal cancer is already a systemic disease at an early stage. Homozygous deletion of the *p16* gene and amplification of the *cyclin D1* gene were observed in xenografts derived from thoracic duct lymph fluid of the ESCC patients [86].

The HSA and Sum cell lines were established from ESCC cells floating in the thoracic duct. These two cell lines had a high migratory ability in vitro and a high lymph node metastatic potential in vivo (Figs. 2, 3; see Table 1) These cells expressed NGF, TIB929 (GTPase superfamily), and osteopontin [51,87,88]. These findings suggest that tumor cells floating in the thoracic duct have been selected from the primary tumor and have adapted to the environment, and may thus have a high metastatic potential.

Only certain cancer cells (so-called cancer stem cells) show the potential for growth at sites of metastasis. Thus, it is necessary to find markers for cancer stem cells or isolate such stem cells [89,90].

Attachment

SLea, SLex, and E-selectin

The carbohydrate determinants sialyl Lewis A and sialyl Lewis X are frequently expressed by human cancer cells and serve as ligands for E-selectin, a cell adhesion molecule from the selectin family that is expressed by vascular endothelial cells. These carbohydrate determinants are involved in the adhesion of cancer cells to the vascular endothelium and thus contribute to hematogenous metastasis of cancer [91].

The expression of sLeA and sLex is correlated with hematogenous recurrence of ESCC and sLeA expression is also correlated with pM (lymph) status. Although neither sLeA nor sLeX expression has a significant influence on the survival of patients with ESCC, logistic regression analysis has demonstrated that increased tumor expression of sLeA and sLeX is a risk factor for hematogenous recurrence of ESCC [92].

Serum levels of soluble E-selectin were significantly higher in a hematogenous recurrence group than in a non-hematogenous recurrence group. Logistic regression analysis showed that high serum soluble E-selectin levels, lymph node metastasis, and intraepithelial spread were associated with postoperative hematogenous recurrence of ESCC [93].

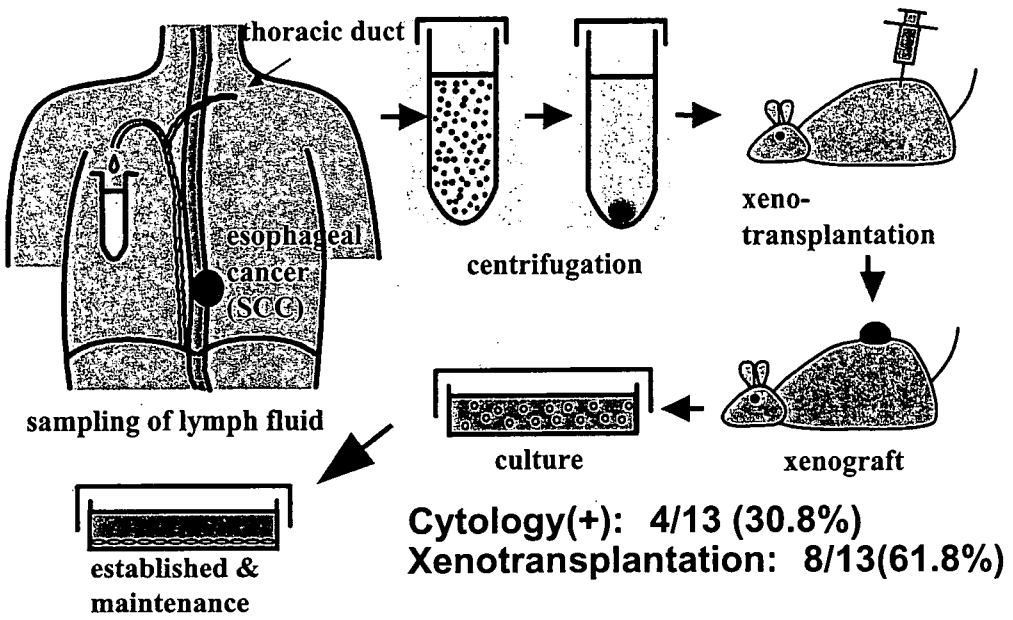

Fig. 2. Establishment of cancer cell lines derived from thoracic duct lymph

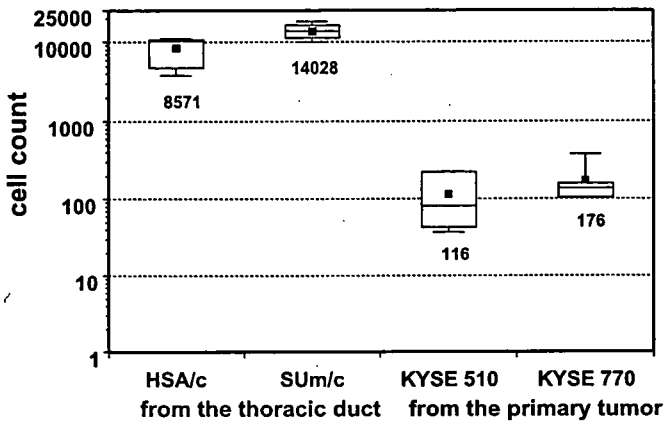

Fig. 3. Migration assay. The HSA and Sum cell lines were established from esophageal squamous cell carcinoma (ESCC) cells floating in the thoracic duct. These two cell lines had a higher migration ability compared with cells from the primary tumor

Angiogenesis

VEGF

Vascular endothelial growth factor (VEGF) has an influence on the growth of malignant tumors by promoting angiogenesis [94]. A significant correlation has been found between VEGF expression and lymph node metastasis in patients with ESCC. The average microvessel density (MVD) shows a correlation with VEGF expression [95], whereas the expression of VEGF, TGF-alpha, platelet-derived endothelial cell growth factor (PD-ECGF), and fibroblast growth factor (FGF) is significantly correlated with lymph node metastasis [96,97].

VEGF-C plays a key role in the process of lymphangiogenesis [94]. Four of five human esophageal carcinoma cell lines were found to constitutively express VEGF-C mRNA [98]. When resected specimens were examined, VEGF-C mRNA was detected in tumor tissues, but not in normal mucosa, by reverse transcriptase-polymerase chain reaction (RT-PCR). There was a significant relationship between VEGF-C and flt-4 mRNA expression. However, VEGF-C was detected in the cytoplasm of many cancer cells and stromal cells whereas Flt-4 was mainly expressed by the endothelial cells of lymphatics. Normal and dysplastic esophageal squamous epithelium shows little or no cytoplasmic staining for VEGF-C, whereas VEGF-C expression is correlated with venous invasion, lymphatic invasion, and lymph node metastasis. The vessel count was found to be significantly higher in VEGF-C-positive tumors than in VEGF-C-negative tumors [98]. Furthermore, VEGF-C overexpression by ESCC with submucosal invasion is a high risk factor for lymph node metastasis (including micrometastasis) [99].

HIF

Hypoxia-inducible factor (HIF)-1-alpha is a transcription factor that regulates the transcription of genes associated with cell proliferation and angiogenesis [100]. HIF-1-alpha was expressed by all five ESCC cell lines that were tested, and HIF-1-alpha expression was found to show a correlation with lymphatic invasion and VEGF-C expression [101]. Furthermore, HIF-1-alpha expression was correlated with both lymph node metastasis and distant metastasis [102].

Other factors

Osteopontin (OPN) is a secreted integrin-binding glycophosphoprotein that is thought to have a role in the metastasis of malignant tumors [103]. cDNA microarray analysis has revealed that high levels of osteopontin mRNA expression are associated with poor survival in ESCC patients. In an immunohistochemical study, osteopontin protein ex-

Table 1. Frequency of lymph node (LN) metastasis by esophageal squamous cell carcinoma (ESCC) cell lines transplanted into nude mice

	Cells	Left popliteal	Left inguinal	Paraaortic	Others	Total
Thoracic duct	SUm/c	5/9	0/9	3/9	0/9	6/9
	HSA/c	8/9	0/9	1/9	0/9	8/9
Primary tumor	KYSE510	1/10	0/10	0/10	0/10	1/10
	KYSE770	0/9	0/9	0/9	0/9	0/9

Data represent mice/mice

	LN metastasis (–)	LN metastasis (+)	Total
Cells from the thoracic duct	4	14	18
Cells from the primary tumor	18	1	19
Total	22	15	37

$P < 0.0001$

Cell lines derived from thoracic duct lymph achieved a significantly higher rate of lymph node metastasis compared with cell lines from the primary tumor

pression was found to be associated with distant lymph node metastasis [88,104]. Thirty tumor cell lines showed overexpression of osteopontin protein compared with a normal esophageal epithelial cell line [88]. An inducible shRNA vector targeting osteopontin caused downregulation of osteopontin expression and decreased the motility, invasiveness, tumor-forming ability, and lymph node metastatic potential of highly metastatic tumor cells [88]. Furthermore, a high plasma OPN level was associated with lymph node metastasis, but not with the depth of tumor invasion [105].

Evidence has been obtained that an inverse correlation exists between the expression of the cysteine proteinase inhibitor stefin A and malignant progression [106]. Transfection of *stefin A* into an esophageal cancer cell line significantly reduced cathepsin B activity and inhibited the invasion of Matrigel. Overexpression of stefin A caused both in vitro and in vivo delay of growth and significantly inhibited lung metastasis. Transfection with *stefin A* caused a dramatic reduction of factor VIII staining in the xenograft tumors of mice [107]. Furthermore, cystatin B (one of the cysteine proteinase inhibitors that mainly inhibits cathepsin L) has been identified in ESCC, and reduced expression of cystatin B in esophageal carcinoma tissue is associated with lymph node metastasis [108]. Thus, cathepsins B and L may have a role in the metastasis of esophageal cancer.

The metastasis-associated protein MTA1 [109], PGP9.5/UCHL1 [110], elongation factor-1 (EF-1) delta [111], and fragile histidine triad (FHIT) genes [112] have also been reported to have a role in lymph node metastasis, but detailed analysis has not been done yet.

Chromosomal changes

Chromosomal analysis has shown that loss of heterozygosity (LOH) on 13q is exclusively associated with lymph node metastasis and with a poor prognosis of esophageal squamous cell carcinoma [113]. Allelic loss of at least one marker is observed in 56.7% of tumors, and lymph node metastasis is significantly correlated with LOH. Loss at D13S260, D13S171, and D13S267 on 13q12-13 is frequently observed, and LOH at D13S171 shows a significant correlation with lymph node metastasis. Thus, allelic loss at 13q12-13 is closely associated with lymph node metastasis, suggesting that unidentified tumor suppressor gene(s) in this region might be involved [113]. Absence of gain of 8q24 and/or 20q12-qter has also been reported to be associated with freedom from lymph node metastasis in patients with superficial ESCC [114]. However, the actual genes affected at these loci have not been identified yet.

Gene expression profile

Stage-dependent gene expression

Sato et al. examined the expression of VEGF, MMP9, and E-cadherin in the early stage of lymph node metastasis (cancer cells occupying <50% of the lymph node) or the late stage (≥50% occupied) [115]. VEGF expression was downregulated in the late stage of lymph node metastasis whereas MMP-9 expression was elevated in the early stage. E-cadherin was expression is somewhat increased in the early stage but was suppressed again in the late stage of metastasis. In summary, the expression of VEGF, MMP-9, and E-cadherin changes during the process of lymph node metastasis by esophageal cancer, and the pattern of change is different for each molecule [115]. There is a complex network of genes involved in the process of tumor metastasis, and the role of each gene changes in importance at various points. Such changes of gene expression need to be observed and defined in more detail.

Global gene expression profiling

Microarray technology allows us to assess the global gene expression profile of circulating tumor cells. Kan et al. examined the gene expression profile of 28 primary ESCCs with an 8000 cDNA microarray chip [116]. Lymph node metastasis-related genes were extracted with significance

analysis of microarrays (SAM) software, and an artificial neural network model was found to predict lymph node metastasis most accurately with 60 clones. The highest accuracy for predicting lymph node metastasis by this method was 77% (10/13) of new patients who were not used for gene selection by SAM analysis and 86% (24/28) overall (sensitivity, 15/17, 88%; specificity, 9/11, 82%) [116]. This system was also able to predict micrometastasis. Comparison of esophageal cancers with and without lymph node metastasis by SAM analysis did not detect any genes that have been reported to show a relationship to lymph node metastasis of esophageal cancer, such as extracellular matrix-degrading enzymes, cytoskeletal and adhesion proteins, or growth factors. However, some genes related to immunity, such as cytokines, and some apoptosis-related molecules were detected. This finding implies that the key factor involved in lymph node metastasis may be the interaction between invasive cancer cells and their microenvironment or host antitumor immunity [116].

Tamoto et al. identified 71 of 1289 cancer-related genes for which expression was correlated with the tumor stage [117]. In the case of lymph node metastasis, 44 genes showed predictive value. After training in classification with the selected features, tumor stage and lymph node metastasis were predicted in 18 cases used for validation with an accuracy of 94.4% and 88.9%, respectively [117].

Another study showed that 4155 genes were biologically significant in both ESCC and noncancerous esophageal tissue by Present Call analysis (hybridization by Affymetrix) [118]. A supervised learning method was used to select genes responsible for the development of ESCC. Intriguingly, there was no overlap between the 48 genes related to lymph node metastasis of pT1 tumors and the 30 genes related to lymph node metastasis of pT2-4 tumors, suggesting that ESCCs with different levels of invasiveness expressed different genes linked to lymph node metastasis. This result suggests that the depth of invasion must be considered when attempting to predict nodal metastasis of ESCC from the gene expression profile [118].

In an experimental study, the expression profile of 9206 genes in T.Tn-AT1 cells (a metastasizing cell line) and T. Tn cells (a nonmetastasizing cell line) was compared by cDNA microarray analysis [119], and only 34 genes showed a more than threefold difference in expression. Among these 34 genes, the expression of 8 genes (*KAL1, HPGD, NDN, REG1A, CXCR4, SPOCK, DIAPH2,* and *AIF1*) was downregulated, whereas the expression of 1 gene (*VNN2*) was upregulated in the metastasizing cells [119]. However, this result for CXCR4 is not consistent with recent clinical data [78].

These findings suggest that global gene expression profiling may be able to predict lymph node metastasis of ESCC but the genes selected have differed among several studies and further examination is needed to assess the clinica implications.

Conclusion

A dynamic multistep process controls the establishment o metastasis of ESCC (Fig. 4, Table 2). Prediction of metas tasis is the first step in selecting the optimum treatmen MMP inhibitors might be a possible treatment optior whereas the results of microarray analysis suggested tha activation of immune surveillance may be another trea ment strategy for esophageal cancer. Finally, shRNA de creased the metastasis of esophageal cancer in vitro, s molecular targeting could perhaps be employed to contr metastasis of ESCC.

Acknowledgments This work was partly supported by a Grant-in-A from the Japanese Ministry of Education, Science and Culture (Gra 17390363).

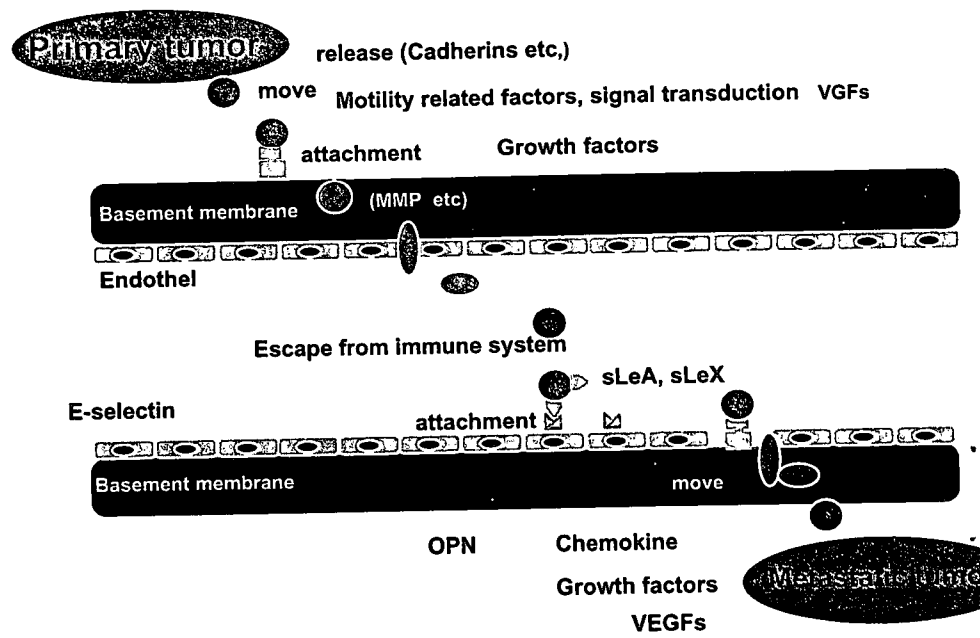

Fig. 4. Multistep genetic changes during the process of metastasis of ESCC

Table 2. Genes related to metastasis of ESCC

Component	Function	Type of metastasis	References
Adhesion molecule1			
E-cadherin	Cell–cell adhesion	LN metastasis	6, 7, 8, 10, 11, 12
Alpha-catenin	Cell–cell adhesion	LN metastasis	10
Slug	Cell–cell adhesion	LN metastasis	17
TSLC1/IGSF4	Cell–cell adhesion	LN metastasis	19
Thrombomodulin	Cell–cell adhesion, anticoagulant	LN metastasis	21
Desmoglein	Cell–cell adhesion	LN metastasis	23
FAK	Cellular focal adhesion	LN metastasis	25
Attachment			
SLex, sLea	Attachment between tumor and endothelial cells	Hematogenous metastasis	92
E-selectin	Attachment between tumor and endothelial cells	Hematogenous metastasis	93
Matrix metalloproteinase			
MMP	Degradation of extracellular matrix (ECM)	LN metastasis	27, 28, 29, 30, 31, 32
TIMP	Degradation of ECM	LN metastasis	33, 34
Proliferation			
Cyclin D1	Regulation of cell cycle	Hematogenous metastasis	37, 38, 39, 86
p16/CDKN2	Regulation of cell cycle	LN metastasis	38, 86
ODC Aurora	Biosynthesis of polyamins	LN metastasis	42
A/STK15/BTAK	Chromosomal distribution	LN metastasis	45
Growth factor			
EGFR	Cell growth	LN metastasis	48
TGF-alpha	Cell growth, angiogenesis	LN metastasis	96
VEGF-A	Neovascularization	LN metastasis	95, 96
VEGF-C	Lymph angiogenesis	LN metastasis	98, 99, 101
PD-ECGF, PynPase	Angiogenesis, 5-converting enzyme	LN metastasis	96, 97
HIF	Angiogenesis	LN metastasis	101, 102
NGF	Cell growth	LN metastasis	51
Motility			
Caveolin-1	Plasma membrane, multifunction	LN metastasis	67
MRP1, KAI1	Cell motility	LN metastasis	75
Fascin	Actin-binding protein	LN metastasis	69
AMF	Autocrine and motility	LN metastasis	71
Laminin-5-gamma-2	Extracellular matrix protein	LN metastasis	48
Chemokine			
CCR7	Chemotaxis	LN metastasis	77
CXCR4	Chemotaxis	LN metastasis	78, 119
Signal transduction			
smad	Signal transduction	LN metastasis	56
Grb7	Signal transduction	LN metastasis	54
RhoGTP	Signal transduction	LN metastasis	60
Activin-beta A	Signal transduction	LN metastasis	58
Shh, Gli	Signal transduction	LN metastasis	65
Others			
OPN	Multifunction	LN metastasis	88, 104, 105
Cathepsin B, L	Cyctein protease	LN metastasis	107, 108
13q LOH	Unknown	LN metastasis	113
8q24, 20q12-qter gain	Unknown	LN metastasis	114

References

1. Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancer in 1990. Int J Cancer 1990;80:827–41.
2. Pisani P, Parkin DM, Bray F, Ferlay J. Estimates of the worldwide mortality from 25 cancers in 1990. Int J Cancer 1999;83:18–29.
3. Steeg PS. Tumor metastasis: mechanistic insights and clinical challenges. Nat Med 2006;12:895–904.
4. Bashyam MD. Understanding cancer metastasis. Cancer (Phila) 2002;94:1821–9.
5. Takeichi M. Cadherins: a molecular family important in selective cell-cell adhesion. Annu Rev Biochem 1990;59:237–52.
6. Doki Y, Shiozaki H, Tahara H, Inoue M, Oka H, Iihara K, et al. Correlation between E-cadherin expression and invasiveness in vitro in a human esophageal cancer cell line. Cancer Res 1993;53: 3421–6.
7. Shiozaki H, Kadowaki T, Doki Y, Inoue M, Tamura S, Oka H, et al. Effect of epidermal growth factor on cadherin-mediated adhesion in a human oesophageal cancer cell line. Br J Cancer 1995;71:250–8.
8. Tamura S, Shiozaki H, Miyata M, Kadowaki T, Inoue M, Matsui S, et al. Decreased E-cadherin expression is associated with haematogenous recurrence and poor prognosis in patients with squamous cell carcinoma of the oesophagus. Br J Surg 1996;83: 1608–14.
9. Nagafuchi A, Takeichi M, Tsukita S. The 102kd cadherin-associated protein: similarity to vinculin and posttranscriptional regulation of expression. Cell 1991;65:849–57.
10. Kadowaki T, Shiozaki H, Inoue M, Tamura S, Oka H, Doki Y, et al. E-cadherin and alpha-catenin expression in human esophageal cancer. Cancer Res 1994;54:291–6.
11. Research Committee on Malignancy of Esophageal Cancer, Japanese Society for Esophageal Diseases. Prognostic significance of cyclin D1 and E-cadherin in patients with esophageal squamous

cell carcinoma: multiinstitutional retrospective analysis. J Am Coll Surg 2001;192:708–18.
12. Shiozaki H, Doki Y, Yamana H, Isono K. A multi-institutional study of immunohistochemical investigation for the role of cyclin D1 and E-cadherin in superficial squamous cell carcinoma of the esophagus. J Surg Oncol 2002;79:166–73.
13. Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, et al. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol 2000;2:76–83.
14. Batlle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, et al. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol 2000;2: 84–9.
15. Takeno S, Noguchi T, Fumoto S, Kimura Y, Shibata T, Kawahara K. E-cadherin expression in patients with esophageal squamous cell carcinoma: promoter hypermethylation, Snail overexpression, and clinicopathologic implications. Am J Clin Pathol 2004;12: 78–84.
16. Hajra KM, Chen DY, Fearon ER. The SLUG zinc-finger protein represses E-cadherin in breast cancer. Cancer Res 2002;62: 1613–8.
17. Uchikado Y, Natsugoe S, Okumura H, Setoyama T, Matsumoto M, Ishigami S, et al. Slug expression in the E-cadherin preserved tumors is related to prognosis in patients with esophageal squamous cell carcinoma. Clin Cancer Res 2005;11:1174–80.
18. Kuramochi M, Fukuhara H, Nobukuni T, Kanbe T, Maruyama T, Ghosh HP, et al. TSLC1 is a tumor-suppressor gene in human non-small-cell lung cancer. Nat Genet 2001;27:427–30.
19. Ito T, Shimada Y, Hashimoto Y, Kaganoi J, Kan T, Watanabe G, et al. Involvement of TSLC1 in progression of esophageal squamous cell carcinoma. Cancer Res 2003;63:6320–6.
20. Esmon CT. Protein C. Prog Hemost Thromb 1984;7:25–54.
21. Tezuka Y, Yonezawa S, Maruyama I, Matsushita Y, Shimizu T, Obama H, et al. Expression of thrombomodulin in esophageal squamous cell carcinoma and its relationship to lymph node metastasis. Cancer Res 1995;55:4196–200.
22. Pasdar M, Nelson WJ. Regulation of desmosome assembly in epithelial cells: kinetics of synthesis, transport, and stabilization of desmoglein I, a major protein of the membrane core domain. J Cell Biol 1989;109:163–77.
23. Natsugoe S, Aikou T, Shimada M, Kumanohoso T, Tezuka Y, Sagara M, et al. Expression of desmoglein I in squamous cell carcinoma of the esophagus. J Surg Oncol 1994;57:105–10.
24. Guan JL, Shalloway D. Regulation of focal adhesion-associated protein tyrosine kinase by both cellular adhesion and oncogenic transformation. Nature (Lond) 1992;358:690–2.
25. Miyazaki T, Kato H, Nakajima M, Sohda M, Fukai Y, Masuda N, et al. FAK overexpression is correlated with tumour invasiveness and lymph node metastasis in oesophageal squamous cell carcinoma. Br J Cancer 2003;89:140–5.
26. Khokha R, Denhardt DT. Matrix metalloproteinases and tissue inhibitor of metalloproteinases: a review of their role in tumorigenesis and tissue invasion. Invasion Metastasis 1989;9:391–405.
27. Shima I, Sasaguri Y, Kusukawa J, Yamana H, Fujita H, Kakegawa T, et al. Production of matrix metalloproteinase-2 and metalloproteinase-3 related to malignant behavior of esophageal carcinoma. A clinicopathologic study. Cancer (Phila) 1992;70:2747–53.
28. Yamashita K, Mori M, Shiraishi T, Shibuta K, Sugimachi K. Clinical significance of matrix metalloproteinase-7 expression in esophageal carcinoma. Clin Cancer Res 2000;6:1169–74.
29. Ding Y, Shimada Y, Gorrin-Rivas MJ, Itami A, Li Z, Hong T, et al. Clinicopathological significance of human macrophage metalloelastase expression in esophageal squamous cell carcinoma. Oncology 2002;63:378–84.
30. Etoh T, Inoue H, Yoshikawa Y, Barnard GF, Kitano S, Mori M. Increased expression of collagenase-3 (MMP-13) and MT1-MMP in oesophageal cancer is related to cancer aggressiveness. Gut 2000;47:50–6.
31. Yamashita K, Tanaka Y, Mimori K, Inoue H, Mori M. Differential expression of MMP and uPA systems and prognostic relevance of their expression in esophageal squamous cell carcinoma. Int J Cancer 2004;110:201–7.
32. Yamamoto H, Vinitketkumnuen A, Adachi Y, Taniguchi H, Hirata T, Miyamoto N, et al. Association of matrilysin-2 (MMP-26) expression with tumor progression and activation of MMP-9 in esophageal squamous cell carcinoma. Carcinogenesis (Oxf) 2004;25:2353–60.
33. Mori M, Mimori K, Sadanaga N, Inoue H, Tanaka Y, Mafune K, et al. Prognostic impact of tissue inhibitor of matrix metalloproteinase-1 in esophageal carcinoma. Int J Cancer 2000;88:575–8.
34. Miyazaki T, Kato H, Nakajima M, Faried A, Takita J, Sohda M, et al. An immunohistochemical study of TIMP-3 expression in oesophageal squamous cell carcinoma. Br J Cancer 2004;91: 1556–60.
35. Kanda Y, Nishiyama Y, Shimada Y, Imamura M, Nomura H, Hiai H, et al. Analysis of gene amplification and overexpression in human esophageal-carcinoma cell lines. Int J Cancer 1994;58: 291–7.
36. Nakagawa H, Zukerberg L, Togawa K, Meltzer SJ, Nishihara T, Rustgi AK. Human cyclin D1 oncogene and esophageal squamous cell carcinoma. Cancer (Phila) 1995;76:541–9.
37. Shinozaki H, Ozawa S, Ando N, Tsuruta H, Terada M, Ueda M, et al. Cyclin D1 amplification as a new predictive classification for squamous cell carcinoma of the esophagus, adding gene information. Clin Cancer Res 1996;2:1155–61.
38. Takeuchi H, Ozawa S, Ando N, Shih CH, Koyanagi K, Ueda M, et al. Altered p16/MTS1/CDKN2 and cyclin D1/PRAD-1 gene expression is associated with the prognosis of squamous cell carcinoma of the esophagus. Clin Cancer Res 1997;3:2229–36.
39. Shimada Y, Imamura M, Watanabe G, Uchida S, Harada H, Makino T, et al. Prognostic factors of oesophageal squamous cell carcinoma from the perspective of molecular biology. Br J Cancer 1999;80:1281–8.
40. Pegg AE. Regulation of ornithine decarboxylase. J Biol Chem 2006;28:14529–32.
41. Kubota S, Kiyosawa H, Nomura Y, Yamada T, Seyama Y. Ornithine decarboxylase overexpression in mouse 10T1/2 fibroblasts: cellular transformation and invasion. J Natl Cancer Inst 1997;89: 567–71.
42. Mafune K, Tanaka Y, Mimori K, Mori M, Takubo K, Makuuchi M. Increased expression of ornithine decarboxylase messenger RNA in human esophageal carcinoma. Clin Cancer Res 1999;5:4073–8.
43. Zhou H, Kuang J, Zhong L, Kuo WL, Gray JW, Sahin A, et al. Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. Nat Genet 1998;20: 189–93.
44. Sen S, Zhou H, Zhang RD, Yoon DS, Vakar-Lopez F, Ito S, et al. Amplification/overexpression of a mitotic kinase gene in human bladder cancer. J Natl Cancer Inst 2002;94(17):1320–9.
45. Tanaka E, Hashimoto Y, Ito T, Okumura T, Kan T, Watanabe G, et al. The clinical significance of Aurora-A/STK15/BTAK expression in human esophageal squamous cell carcinoma. Clin Cancer Res 2005;11:1827–34.
46. Ozawa S, Ueda M, Ando N, Shimizu N, Abe O. Prognostic significance of epidermal growth factor receptor in esophageal squamous cell carcinomas. Cancer (Phila) 1989;63:2169–73.
47. Yamamoto T, Kamata N, Kawano H, Shimizu S, Kuroki T, Toyoshima K, et al. High incidence of amplification of the epidermal growth factor receptor gene in human squamous carcinoma cell lines. Cancer Res 1986;46:414–6.
48. Fukai Y, Masuda N, Kato H, Fukuchi M, Miyazaki T, Nakajima M, et al. Correlation between laminin-5 gamma 2 chain and epidermal growth factor receptor expression in esophageal squamous cell carcinomas. Oncology 2005;69:71–80.
49. Dolle L, El Yazidi-Belkoura I, Adriaenssens E, Nurcombe V, Hondermarck H. Nerve growth factor overexpression and autocrine loop in breast cancer cells. Oncogene 2003;22:5592–601.
50. Davidson B, Reich R, Lazarovici P, Nesland JM, Skrede M, Risberg B, et al. Expression and activation of the nerve growth factor receptor TrkA in serous ovarian carcinoma. Clin Cancer Res 2003;9:2248–59.
51. Tsunoda S, Okumura T, Ito T, Mori Y, Soma T, Watanabe G, et al. Significance of nerve growth factor overexpression and its autocrine loop in oesophageal squamous cell carcinoma. Br J Cancer 2006;95:322–30.

52. Margolis B, Silvennoinen O, Comoglio F, Roonprapunt C, Skolnik E, Ullrich A, et al. High-efficiency expression/cloning of epidermal growth factor-receptor-binding proteins with Src homology 2 domains. Proc Natl Acad Sci U S A 1992;89:8894–8.
53. Manser J, Roonprapunt C, Margolis B. *C. elegans* cell migration gene mig-10 shares similarities with a family of SH2 domain proteins and acts cell nonautonomously in excretory canal development. Dev Biol 1997;184:150–64.
54. Tanaka S, Sugimachi K, Kawaguchi H, Saeki H, Ohno S, Wands JR. Grb7 signal transduction protein mediates metastatic progression of esophageal carcinoma. J Cell Physiol 2000;183:411–5.
55. Kretzschmar M, Massague J. SMADs: mediators and regulators of TGF-beta signaling. Curr Opin Genet Dev 1998;8:103–11.
56. Natsugoe S, Xiangming C, Matsumoto M, Okumura H, Nakashima S, Sakita H, et al. Smad4 and transforming growth factor beta1 expression in patients with squamous cell carcinoma of the esophagus. Clin Cancer Res 2002;8:1838–42.
57. Schuldiner M, Yanuka O, Itskovitz-Eldor J, Melton DA, Benvenisty N. Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. Proc Natl Acad Sci U S A 2000;97:11307–12.
58. Yoshinaga K, Mimori K, Yamashita K, Utsunomiya T, Inoue H, Mori M. Clinical significance of the expression of activin A in esophageal carcinoma. Int J Oncol 2003;22:75–80.
59. Schmitz AA, Govek EE, Bottner B, Van Aelst L. Rho GTPases: signaling, migration, and invasion. Exp Cell Res. 2000;261:1–12.
60. Faried A, Faried LS, Kimura H, Nakajima M, Sohda M, Miyazaki T, et al. RhoA and RhoC proteins promote both cell proliferation and cell invasion of human oesophageal squamous cell carcinoma cell lines in vitro and in vivo. Eur J Cancer 2006;42:1455–65.
61. Ruiz i Altaba A, Palma V, Dahmane N. Gli and Hedgehog in cancer: tumors, embryos and stem cells. Nat Rev Neurosci 2002;3:24–33.
62. Magliano MP, Hebrok M. Hedgehog signaling in cancer formation and maintenance. Nat Rev Cancer 2003;3:903–11.
63. Berman DM, Karhadkar SS, Maltra A, Montes De Oca R, Gerstenblith MR, Briggs K, et al. Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. Nature (Lond) 2003;425:846–51.
64. Karhadkar SS, Bova GS, Abdallah N, Dhara S, Gardner D, Maitra A, et al. Hedgehog signalling in prostate regeneration, neoplasia and metastasis. Nature (Lond) 2004;431:707–12.
65. Mori Y, Okumura T, Tsunoda S, Sakai Y, Shimada Y. The Gli-1 expression is associated with lymph node metastasis and tumor progression in esophageal squamous cell carcinoma. Oncology 2006;70:378–89.
66. Williams TM, Medina F, Badano I, Hazan RB, Hutchinson J, Muller WJ, et al. Caveolin-1 gene disruption promotes mammary tumorigenesis and dramatically enhances lung metastasis in vivo. Role of Cav-1 in cell invasiveness and matrix metalloproteinase (MMP-2/9) secretion. J Biol Chem 2004;279:51630–46.
67. Kato K, Hida Y, Miyamoto M, Hashida H, Shinohara T, Itoh T, et al. Overexpression of caveolin-1 in esophageal squamous cell carcinoma correlates with lymph node metastasis and pathologic stage. Cancer (Phila) 2002;94:929–33.
68. Hashimoto Y, Skacel M, Adams JC. Roles of fascin in human carcinoma motility and signaling: prospects for a novel biomarker? Int J Biochem Cell Biol 2005;37:1787–804.
69. Hashimoto Y, Ito T, Inoue H, Okumura T, Tanaka E, Tsunoda S, et al. Prognostic significance of fascin overexpression in human esophageal squamous cell carcinoma. Clin Cancer Res 2005;11: 2597–605.
70. Nabi IR, Watanabe H, Raz A. Autocrine motility factor and its receptor: role in cell locomotion and metastasis. Cancer Metastasis Rev 1992;11:5–20.
71. Maruyama K, Watanabe H, Shiozaki H, Takayama T, Gofuku J, Yano H, et al. Expression of autocrine motility factor receptor in human esophageal squamous cell carcinoma. Int J Cancer 1995;64:316–21.
72. Patarroyo M, Tryggvason K, Virtanen I. Laminin isoforms in tumor invasion, angiogenesis and metastasis. Semin Cancer Biol 2002;12:197–207.
73. Ikeyama S, Koyama M, Yamaoko M, Sasada R, Miyake M. Suppression of cell motility and metastasis by transfection with human motility-related protein (MRP-1/CD9) DNA. J Exp Med 1993;177:1231–7.
74. Maecker HT, Todd SC, Levy S. The tetraspanin superfamily: molecular facilitators. FASEB J 1997;11:428–42.
75. Uchida S, Shimada Y, Watanabe G, Li ZG, Hong T, Miyake M, et al. Motility-related protein (MRP-1/CD9) and KAI1/CD82 expression inversely correlate with lymph node metastasis in oesophageal squamous cell carcinoma. Br J Cancer 1999;79:1168–73.
76. Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, et al. Involvement of chemokine receptors in breast cancer metastasis. Nature (Lond) 2001;410:50–6.
77. Ding Y, Shimada Y, Maeda M, Kawabe A, Kaganoi J, Komoto I, et al. Association of CC chemokine receptor 7 with lymph node metastasis of esophageal squamous cell carcinoma. Clin Cancer Res 2003;9:3406–12.
78. Kaifi JT, Yekebas EF, Schurr P, Obonyo D, Wachowiak R, Busch P, et al. Tumor-cell homing to lymph nodes and bone marrow and CXCR4 expression in esophageal cancer. J Natl Cancer Inst 2005;97:1840–7.
79. Izbicki JR, Hosch SB, Pichlmeier U, Rehders A, Busch C, Niendorf A, et al. Prognostic value of immunohistochemically identifiable tumor cells in lymph nodes of patients with completely resected esophageal cancer. N Engl J Med 1997;337:1188–94.
80. Sato F, Shimada Y, Li Z, Watanabe G, Maeda M, Imamura M. Lymph node micrometastasis and prognosis in patients with oesophageal squamous cell carcinoma. Br J Surg 2001;88:426–32.
81. Scheunemann P, Izbicki IR, Pantel K. Tumorigenic potential of apparently tumor-free lymph nodes. N Engl J Med 1999; 340:1687.
82. Nagatani S, Shimada Y, Sato F, Watanabe G, Maeda M, Kaganoi J, et al. Expression of E-cadherin, cyclin D1, matrix metalloproteinase 9, and vascular endothelial growth factor in primary tumor of esophageal squamous cell carcinoma does not predict lymph node micrometastasis. Esophagus 2005;2:71–5.
83. Mori M, Mimori K, Ueo H, Tsuji K, Shiraishi T, Barnard GF, et al. Clinical significance of molecular detection of carcinoma cells in lymph nodes and peripheral blood by reverse transcription-polymerase chain reaction in patients with gastrointestinal or breast carcinomas. J Clin Oncol 1998;16:128–32.
84. Nakashima S, Natsugoe S, Matsumoto M, Miyazono F, Nakajo A, Uchikura K, et al. Clinical significance of circulating tumor cells in blood by molecular detection and tumor markers in esophageal cancer. Surgery (St. Louis) 2003;133:162–9.
85. Kaganoi J, Shimada Y, Kano M, Okumura T, Watanabe G, Imamura M. Detection of circulating oesophageal squamous cancer cells in peripheral blood and its impact on prognosis. Br J Surg 2004;91:1055–60.
86. Watanabe H, Tachimori Y, Katon H, Nakanishi K, Ochiai A, Gyotoku M, et al. Tumor progression of cancer cells derived from lymph fluid of thoracic duct for esophageal cancer patients by orthotopic transplantation on nude rat. Biotherapy 1996;10: 1332–7.
87. Nakaji T, Kataoka TR, Watabe K, Nishiyama K, Nojima H, Shimada Y, et al. A new member of the GTPase superfamily that is upregulated in highly metastatic cells. Cancer Lett 1999;147: 139–47.
88. Ito T, Hashimoto Y, Tanaka E, Kan T, Tsunoda S, Sato F, et al. An inducible short-hairpin RNA vector against osteopontin reduces metastatic potential of human esophageal squamous cell carcinoma in vitro and in vivo. Clin Cancer Res 2006;12: 1308–16.
89. Haraguchi N, Utsunomiya T, Inoue H, Tanaka F, Mimori K, Barnard GF, et al. Characterization of a side population of cancer cells from human gastrointestinal system. Stem Cells 2006;24: 506–13.
90. Ban S, Ishikawa K, Kawai S, Koyama-Saegusa K, Ishikawa A, Shimada Y, et al. Potential in a single cancer cell to produce heterogeneous morphology, radiosensitivity and gene expression. J Radiat Res (Tokyo) 2005;46:43–50.
91. Kannagi R. Carbohydrate-mediated cell adhesion involved in hematogenous metastasis of cancer. Glycoconj J 1997;14:577–84.
92. Makino T, Shimada Y, Maeda M, Komoto I, Imamura M. Carbohydrate antigens as a risk factor for hematogenous recurrence

of esophageal squamous cell carcinoma patients. Oncol Rep 2001;8:981–5.
93. Shimada Y, Maeda M, Watanabe G, Imamura M. High serum soluble E-selectin levels are associated with postoperative haematogenic recurrence in esophageal squamous cell carcinoma patients. Oncol Rep 2003;10:991–5.
94. Roy H, Bhardwaj S, Yla-Herttuala S. Biology of vascular endothelial growth factors. FEBS Lett 2006;580:2879–87.
95. Uchida S, Shimada Y, Watanabe G, Tanaka H, Shibagaki I, Miyahara T, et al. In oesophageal squamous cell carcinoma vascular endothelial growth factor is associated with p53 mutation, advanced stage and poor prognosis. Br J Cancer 1998;77:1704–9.
96. Li Z, Shimada Y, Uchida S, Maeda M, Kawabe A, Mori A,et al. TGF-alpha as well as VEGF, PD-ECGF and bFGF contribute to angiogenesis of esophageal squamous cell carcinoma. Int J Oncol 2000;17:453–60.
97. Yamagata M, Mori M, Mimori K, Mafune KI, Tanaka Y, Ueo H, et al. Expression of pyrimidine nucleoside phosphorylase mRNA plays an important role in the prognosis of patients with oesophageal cancer. Br J Cancer 1999;79:565–9.
98. Kitadai Y, Amioka T, Haruma K, Tanaka S, Yoshihara M, Sumii K, et al. Clinicopathological significance of vascular endothelial growth factor (VEGF)-C in human esophageal squamous cell carcinomas. Int J Cancer 2001;93:662–6.
99. Matsumoto M, Natsugoe S, Okumura H, Arima H, Yanagita S, Uchikado Y, et al. Overexpression of vascular endothelial growth factor-C correlates with lymph node micrometastasis in submucosal esophageal cancer. J Gastrointest Surg 2006;10:1016–22.
100. Pouyssegur J, Dayan F, Mazure NM. Hypoxia signaling in cancer and approaches to enforce tumour regression. Nature (Lond) 2006;441:437–43.
101. Katsuta M, Miyashita M, Makino H, Nomura T, Shinji S, Yamashita K, et al. Correlation of hypoxia inducible factor-1-alpha with lymphatic metastasis via vascular endothelial growth factor-C in human esophageal cancer. Exp Mol Pathol 2005;78: 123–30.
102. Kurokawa T, Miyamoto M, Kato K, Cho Y, Kawarada Y, Hida Y, et al. Overexpression of hypoxia-inducible-factor 1alpha (HIF-1alpha) in oesophageal squamous cell carcinoma correlates with lymph node metastasis and pathologic stage. Br J Cancer 2003;89: 1042–7.
103. Furger KA, Menon RK, Tuck AB, Bramwell VH, Chambers AF. The functional and clinical roles of osteopontin in cancer and metastasis. Curr Mol Med 2001;1:621–32.
104. Kita Y, Natsugoe S, Okumura H, Matsumoto M, Uchikado Y, Setoyama T, et al. Expression of osteopontin in oesophageal squamous cell carcinoma. Br J Cancer 2006;95:634–8.
105. Shimada Y, Watanabe G, Kawamura J, Soma T, Okabe M, Ito T, et al. Clinical significance of osteopontin in esophageal squamous cell carcinoma: comparison with common tumor markers. Oncology 2005;68:285–92.
106. Sloane BF, Moin K, Krepela E, Rozhin J. Cathepsin B and its endogenous inhibitors: the role in tumor malignancy. Cancer Metastasis Rev 1990;9:333–52.
107. Li W, Ding F, Zhang L, Liu Z, Wu Y, Luo A, et al. Overexpression of stefin A in human esophageal squamous cell carcinoma cells inhibits tumor cell growth, angiogenesis, invasion, and metastasis. Clin Cancer Res 2005;11:8753–62.
108. Shiraishi T, Mori M, Tanaka S, Sugimachi K, Akiyoshi T. Identification of cystatin B in human esophageal carcinoma using differential displays in which the gene expression is related to lymph-node metastasis. Int J Cancer 1998;79:175–8.
109. Toh Y, Kuwano H, Mori M, Nicolson GL, Sugimachi K. Overexpression of metastasis-associated MTA1 mRNA in invasive oesophageal carcinomas. Br J Cancer 1999;79:1723–6.
110. Mandelker DL, Yamashita K, Tokumaru Y, Mimori K, Howard DL, Tanaka Y, et al. PGP9.5 promoter methylation is an independent prognostic factor for esophageal squamous cell carcinoma. Cancer Res 2005;65:4963–8.
111. Ogawa K, Utsunomiya T, Mimori K, Tanaka Y, Tanaka F, Inoue H, et al. Clinical significance of elongation factor-1 delta mRNA expression in oesophageal carcinoma. Br J Cancer 2004;91:282–6.
112. Tzao C, Sun GH, Tung HJ, Hsu HS, Hsu WH, Wang YC, et al. Reduced acetylated histone H4 is associated with promoter methylation of the fragile histidine triad gene in resected esophageal squamous cell carcinoma. Ann Thorac Surg 2006;82:396–401.
113. Harada H, Tanaka H, Shimada Y, Shinoda M, Imamura M, Ishizaki K. Lymph node metastasis is associated with allelic loss on chromosome 13q12-13 in esophageal squamous cell carcinoma. Cancer Res 1999;59:3724–9.
114. Ueno T, Tangoku A, Yoshino S, Abe T, Hayashi H, Toshimitsu H, et al. Prediction of nodal metastasis by comparative genomic hybridization in biopsy specimens from patients with superficial esophageal squamous cell carcinoma. Clin Cancer Res 2003;9:5137–41.
115. Sato F, Shimada Y, Watanabe G, Uchida S, Makino T, Imamura M. Expression of vascular endothelial growth factor, matrix metalloproteinase-9 and E-cadherin in the process of lymph node metastasis in oesophageal cancer. Br J Cancer 1999;80:1366–72.
116. Kan T, Shimada Y, Sato F, Ito T, Kondo K, Watanabe G, et al. Prediction of lymph node metastasis with use of artificial neural networks based on gene expression profiles in esophageal squamous cell carcinoma. Ann Surg Oncol 2004;11:1070–8.
117. Tamoto E, Tada M, Murakawa K, Takada M, Shindo G, Teramoto K, et al. Gene-expression profile changes correlated with tumor progression and lymph node metastasis in esophageal cancer. Clin Cancer Res 2004;10:3629–38.
118. Sato T, Iizuka N, Hamamoto Y, Yoshino S, Abe T, Takeda S, et al. Esophageal squamous cell carcinomas with distinct invasive depth show different gene expression profiles associated with lymph node metastasis. Int J Oncol 2006;28:1043–55.
119. Kawamata H, Furihata T, Omotehara F, Sakai T, Horiuchi H, Shinagawa Y, et al. Identification of genes differentially expressed in a newly isolated human metastasizing esophageal cancer cell line, T.Tn-AT1, by cDNA microarray. Cancer Sci 2003;94 699–706.

Int J Clin Oncol (2007) 12:224–227
DOI 10.1007/s10147-007-0666-x

ORIGINAL ARTICLE

Masashi Kanai* · Shigemi Matsumoto*
Takahumi Nishimura · Yutaka Shimada · Go Watanabe
Toshiyuki Kitano · Akiko Misawa · Hiroshi Ishiguro
Kiyotsugu Yoshikawa · Kazuhiro Yanagihara
Satoshi Teramukai · Michihide Mitsumori
Tsutomu Chiba · Yoshiharu Sakai · Masanori Fukushima

Retrospective analysis of 27 consecutive patients treated with docetaxel/nedaplatin combination therapy as a second-line regimen for advanced esophageal cancer

Received: September 1, 2006 / Accepted: February 2, 2007

Abstract

Background. The aim of this study was to evaluate the efficacy and safety of combination therapy with docetaxel and nedaplatin in advanced esophageal cancer as a second-line regimen in an outpatient setting.

Methods. Twenty-seven consecutive patients with advanced esophageal cancer who received docetaxel/nedaplatin combination therapy as a second-line regimen were retrospectively evaluated. The combination therapy consisted of intravenous administration of docetaxel 30 mg/m^2 and nedaplatin 40 mg/m^2 every 2 weeks.

Results. The patients received a median of 7.4 cycles of treatment (range, 2–25 cycles). No complete response was observed, and 3 of the 27 patients (11%) achieved partial responses. The disease control rate (partial response + stable disease) was 52%. The median survival time (MST) was 11.4 months. Severe hematological adverse events (grade 3–4) were: neutropenia (n = 10; 37%) and anemia (n = 5; 19%); there was neither febrile neutropenia nor grade 3-4 thrombocytopenia. Furthermore, no severe nonhematological adverse events or treatment-related deaths were observed.

Conclusion. Combination therapy of docetaxel with nedaplatin was safe and well tolerated; however, the development of more effective therapy is warranted to improve the prognosis of esophageal cancer.

Key words Docetaxel · Nedaplatin · Esophageal cancer · Chemotherapy

M. Kanai · S. Matsumoto (✉) · T. Nishimura · T. Kitano · A. Misawa · H. Ishiguro · K. Yoshikawa · K. Yanagihara · M. Fukushima
Outpatient Oncology Unit, Kyoto University Hospital, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan
Tel. +81-75-751-4770; Fax +81-75-751-4772
e-mail: motocame@kuhp.kyoto-u.ac.jp

Y. Shimada · G. Watanabe · Y. Sakai
Department of Surgery, Kyoto University Hospital, Kyoto, Japan

S. Teramukai · M. Fukushima
Translational Research Center, Kyoto University Hospital, Kyoto, Japan

M. Mitsumori
Department of Radiation Oncology and Image-Applied Therapy, Kyoto University Hospital, Kyoto, Japan

T. Chiba
Department of Gastroenterology and Hepatology, Kyoto University Hospital, Kyoto, Japan

*These authors contributed equally to this study

Introduction

The incidence of esophageal cancer is increasing in Western countries as well as in Japan. Interestingly, squamous cell carcinoma accounts for more than 90% of esophageal cancers in Japan, while adenocarcinoma has rapidly increased over the past decade and has now become the most common histological type in Western countries.[1,2] In both histological subtypes, early lymphogenic and hematogenous spread is a common feature, resulting in the poor prognosis of this disease.[3] Even patients with the earliest stage disease have a significant risk of recurrence. Thus, locoregional treatment is not sufficient to treat this highly lethal disease, and the development of effective systemic chemotherapy is urgently needed. A variety of chemotherapeutic regimens has been tested until now;[4–6] however, no gold standard chemotherapy regimen has been established for advanced esophageal cancer. At present, a combination of cisplatin and continuous-infusion 5-fluorouracil (5-FU) is one of the most commonly used regimens, with a 25%–35% response rate in metastatic disease.[3,7] The major problem of cisplatin-based chemotherapy lies in its nephrotoxity. The use of this chemotherapy mandates large-volume hydration, which limits the management of this agent in an outpatient setting, and badly hampers the patient's quality of life.

Docetaxel has been proven to show antitumor activity against various cancers, including esophageal cancer.[8–10] Clinical trials of single-agent docetaxel have demonstrated 20%–28% response rates for its use as a second-line regimen in advanced esophageal cancer.[9,10]

Nedaplatin (*cis*-diammine-glycolate platinum) is a second-generation platinum derivative developed in Japan, and several in vitro studies have demonstrated that nedaplatin has equivalent antitumor activity to cisplatin, with less nephrotoxity.[11,12] Consistent with the results of the in vitro studies, nedaplatin in combination with other agents has shown modest antitumor activity for several human tumors, with less nephrotoxicity and gastrointestinal toxicity.[13,14] These reports prompted us to use a combination of docetaxel with nedaplatin as a second-line regimen in patients with advanced esophageal cancer, because pretreated patients have poorer tolerance to second-line chemotherapy, and a less toxic treatment is desirable. Supporting our idea, at the time we started this combination therapy, several Japanese groups were also using docetaxel/nedaplatin combination regimens for treating advanced esophageal cancer, as well as for head and neck cancer. We carried out the present retrospective study to evaluate docetaxel/nedaplatin combination therapy as a second-line regimen in patients with esophageal cancer, and we report our findings here.

Patients and methods

Patients

Twenty-seven consecutive patients who received docetaxel/nedaplatin combination therapy as a second-line regimen at Kyoto University Hospital, between January 2004 and June 2006, were retrospectively analyzed. All the patients had histologically proven esophageal cancer with metastasis. Docetaxel/nedaplatin combination therapy was given if the patient met the following criteria: an Eastern Cooperative Oncology Group (ECOG) performance status of 0–2; adequate baseline bone marrow function (hemoglobin level, 8 g/dl; neutrophil count, 1000/mm^3; and platelet count, 75000/mm^3); adequate hepatic function (total bilirubin level, 1.5 mg/dl; and aspartate aminotransferase and alanine aminotransferase levels, 3.0 the upper limit of normal); adequate renal function (serum creatinine level, 1.5 mg/dl); and written informed consent to receive chemotherapy at our hospital. Toxicities were graded according to the Common Terminology Criteria for Adverse Events v 3.0 (CTCAE v 3.0). Measurable target lesions were assessed by the Response Evaluation Criteria in Solid Tumors (RECIST).[15]

Treatment plan

Docetaxel 30 mg/m^2 (Taxotere; Aventis Pharma, Tokyo, Japan) was infused in 40 min, followed by nedaplatin 40 mg/m^2 (Aqupla; Shionogi Pharma, Tokyo, Japan) over 1.5 h, every 2 weeks. These doses were based on a phase I trial of chemotherapy using docetaxel and nedaplatin in chemotherapy-naïve patients with oral squamous cell carcinoma in Japan.[15] That phase I trial recommended 60 mg/m^2 docetaxel and 100 mg/m^2 nedaplatin every 4 weeks in chemotherapy-naïve patients. Because our treatment was to be repeated every 2 weeks in patients who had had prior chemotherapy, we adjusted the doses of docetaxel and nedaplatin to 30 mg/m^2 and 40 mg/m^2, respectively. Premedication with an H_2-blocker and dexamethasone, as well as post-therapy hydration with 500 ml saline was given for every administration. 5-HT_3 antagonists were added when the oncologist judged it necessary. If the neutrophil count was less than 1000/μl or the platelet count was less than 75000/μl on the day of administration, treatment was adjourned until the count recovered. The dose of docetaxel and nedaplatin was reduced by 20% when the oncologist judged it necessary, according to hematological and other toxicities observed in the previous course. Treatment was continued until disease progression, unacceptable adverse events, patient withdrawal, or death.

Follow-up evaluation

Standard radiological examinations, using computed tomography (CT) scans, were applied for response every 1 to 3 months. Tumor response was assessed based on RECIST.[14] Metastatic lymph nodes within a previous radiation field and the primary lesion were also evaluated. Complete response (CR) was defined as the disappearance of all evidence of cancer, and partial response (PR) as a more than 30% reduction in the sum of the longest diameters of target lesions. Stable disease (SD) was defined as either a less than 30% reduction in the sum of the longest diameters of target lesions, or less than a 20% increase without any new lesions. Progressive disease (PD) was defined as more than a 20% increase in the sum of the longest diameters of target lesions or the appearance of new lesions. The best response achieved during the treatment course was reported.

Statistical methods

Overall survival was measured from the start of the treatment to the date of death. If the patients had not died, overall survival was censored on the last confirmed date of survival. Time to treatment failure (TTF) was measured from the start of the treatment to the date when the treatment was discontinued. The Kaplan–Meier method was used to estimate the overall survival curve. Survival time was censored at the last confirmation date if the patients were alive.

Results

Patient characteristics

Twenty-seven consecutive patients who received docetaxel/nedaplatin combination therapy as a second-line regimen at Kyoto University Hospital were evaluated for response, survival, and toxicity. Patient baseline characteristics are summarized in Table 1. The most common histological type was squamous cell carcinoma ($n = 26$; 96%), and there

Table 1. Patients' baseline characteristics

Characteristics	*n* (%)
Age (years)	
Median	62.5
Range	45–84
Sex	
Male	25 (93)
Female	2 (7)
ECOG performance status	
0	25 (93)
1	2 (7)
2	0
Histology	
Squamous cell carcinoma	26 (96)
Adenocarcinoma	1 (4)
Previous treatment	
Chemoradiotherapy	14 (52)
Chemoradiotherapy surgery	5 (19)
Surgery chemoradiotherapy	3 (11)
Surgery chemotherapy	5 (19)
Evaluated lesions	
Lung	7 (23)
Liver	4 (13)
Lymph nodes	15 (50)
Others	4 (13)

Table 2. Tumor response

No. of patients	Response				Disease control rate (%)
	CR	PR	SD	PD	
27	0	3	11	13	52

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; disease control rate, CR + PR + SD

was one adenocarcinoma case. All patients had a history of prior treatment, consisting of chemoradiotherapy (n = 14), chemoradiotherapy/surgery (n = 5), surgery/chemotherapy (n = 5), and surgery/chemoradiotherapy (n = 3). A combination of cisplatin (70 mg/m^2) on day 1 and continuous-infusion 5-FU (700 mg/m^2 per day on days 1 through 5) had been repeated at 4-week intervals, with or without radiotherapy in 24 patients (89%). No patients had a history of prior chemotherapy containing docetaxel or nedaplatin.

Response and survival

Patients received a median of 7.4 cycles of treatment (range, 2–25 cycles). Response rates are summarized in Table 2. There was no CR, and 3 of the 27 patients (11%) achieved a PR. The disease control rate (PR + SD) was 52%. Twelve patients had measurable lymph nodes within the previous radiation field. Among these 12 patients, only one patient had another target size of lung. The response rates in these 12 patients were as follows; 1PR (8%), 95D (75%) and 2PD (17%) field, PR, SD, and PD were observed in 1/12 (8%), 9/12 (75%), 2/12 (17%), respectively. When this article was submitted, 8 of the 27 patients were still alive. The overall survival of the 27 patients is shown in Fig. 1. The median survival time was 11.4 months (95% confidence interval [CI], 6.8–18.7), the 1-year survival rate was 46.5% (95% CI, 25.0–68.0), and the median time to treatment failure (TTF) was 4.3 months (range, 1.0–20.2 months).

Table 3. Worst grade of toxicity per patient during all cycles: CTCAE v 3.0 grade [No. (%)]

Toxicity	Grade 1	Grade 2	Grade 3	Grade 4
Anemia	10 (37)	8 (30)	4 (15)	1 (4)
Neutropenia	0	2 (7)	7 (26)	3 (11)
Febrile neutropenia	–	–	0	0
Leukopenia	0	9 (33)	7 (26)	0
Thrombocytopenia	7 (26)	1 (4)	0	0
Anorexia	14 (51)	3 (11)	0	0
Fatigue	14 (51)	2 (7)	0	0
Diarrhea	9 (33)	0	0	0
Nausea	5 (19)	2 (7)	0	0
Vomiting	3 (11)	1 (4)	0	0
Neuropathy-sensory	5 (19)	0	0	0

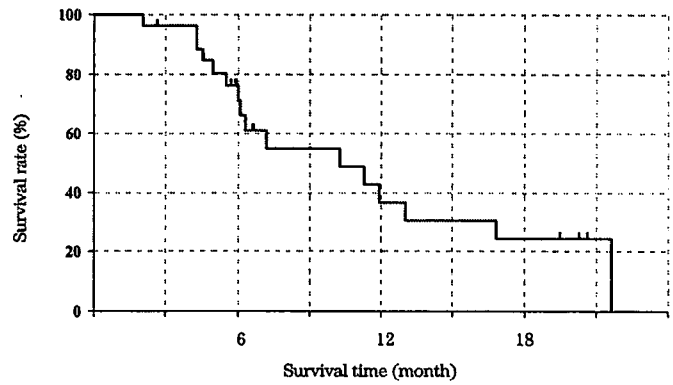

Fig. 1. Overall Survival for 27 patients

Toxicity

The docetaxel/nedaplatin combination therapy was generally safe and well tolerated. Dose reduction was required in 5 of the 27 patients (18.5%). Hematological and nonhematological toxicities are summarized in Table 3. The most common and severe, but reversible, toxicity of this combination therapy was neutropenia. Grade 3 and grade 4 neutropenia were observed in 7 (26%) and 3 (11%) patients, respectively. Febrile neutropenia was not observed in this study. Grade 3–4 anemia was observed in 5 (19%) patients. Anorexia and fatigue were the most common nonhematological toxicities (grade 1–2 anorexia, 62%; grade 1–2 fatigue, 58%). However, neither severe nonhematological adverse events (grade 3–4) nor treatment-related deaths were observed.

Discussion

A number of studies have reported on chemotherapy for advanced esophageal cancer; however, most of these re-

ports evaluated a first-line regimen for chemonaïve patients and only a limited number of studies focused on second-line chemotherapy for advanced esophageal cancer. The low incidence and poor prognosis of esophageal cancer also limit therapeutic trials for this disease. Among the limited number of studies focusing on second-line chemotherapy, several groups evaluated the efficacy of single-agent docetaxel for advanced esophageal cancer. Two independent studies, from France and Japan, reported overall response rates of 28% and 20%, respectively, with single-agent docetaxel at 70–100 mg/m^2 every 3 weeks.[9,10] Because pretreated patients have poorer tolerance to second-line chemotherapy, lower toxicity, as well as efficacy, is an important factor when considering the second-line chemotherapy. By combining nedaplatin at 40 mg/m^2 with docetaxel at 30 mg/m^2 every 2 weeks, we expected less toxicity, with preserved efficacy. In our current study, we observed a partial response (PR) in 3 of the 27 patients (11%), a disease control rate (PR + SD) of 52%, a 1-year survival rate of 46.5% and the MST of 11.4 months.

On the other hand, this combination regimen demonstrated less nephrotoxicity and gastrointestinal toxicity, as we expected. Only 37% of the patients developed grade 3–4 neutropenia and neither febrile neutropenia nor treatment-related deaths were observed in this study. Interestingly, while we were revising this manuscript, Osaka et al.[16] reported the feasibility of docetaxel/nedaplatin combination therapy for patients with recurrent esophageal cancer. Their regimen consisted of docetaxel 30 mg/m^2 and nedaplatin 40 mg/m^2 every 2 weeks, which was completely consistent with the regimen in our study. They evaluated 28 patients with recurrent esophageal cancer who had had prior chemotherapy, and they reported a response rate of 39.3%, with one complete response and an MST of 8.5 months. They also noted that the toxicity was relatively low and that this regimen could be safely managed in an outpatient setting. Table 4 summarizes the results of second-line chemotherapy using docetaxel/nedaplatin combination therapy for advanced esophageal cancer. The docetaxel/nedaplatin combination appears to have a modest effect on advanced esophageal cancer, with less toxicity however, we must be cautious in interpreting these results, because both our study and that of Osaka et al.[16] are retrospective, with a small sample size. Furthermore, because of the lack of phase I studies, the dose-finding method was not scientific in either of the studies, and the lower toxicity observed with this regimen may be attributable to the low dose intensity. However, a recent phase I study from another group (Abe et al.[17]) reported a recommended dose of 30 mg/m^2 docetaxel and 30 mg/m^2 nedaplatin every 2 weeks for advanced esophageal cancer. That report scientifically supports the dose used in our regimen.

Table 4. Results of second-line chemotherapy with a combination of docetaxel/nedaplatin for advanced esophageal cancer

	Our report	Osaka et al.[16]
No. of patients	27	28
Response rate	11%	39%
MST (months)	11.4	8.5
Grade 3–4 leukopenia	7/27 (26%)	2/28 (7.1%)
Grade 3–4 anemia	5/27 (19%)	1/28 (3.6%)
Grade 3–4 thrombocytopenia	0/27 (0%)	0/28 (0%)

References

1. Greenlee RT, Hill-Harmon MB, Murray T, et al. (2001) Cancer statistics. CA Cancer J Clin 51:15–36. Erratum in: CA Cancer J Clin 2001 Mar–Apr; 51:144
2. Foundation for promotion of cancer Research (2001) Cancer Statistics in Japan Editorial Board. Tokyo, Japanese National Cancer Center
3. Enzinger PC, Ilson DH, Kelsen DP (1999) Chemotherapy in esophageal cancer (review). Semin Oncol 26(5 Suppl 15):12–20
4. Ajani JA, Ilson DH, Daugherty K, et al. (1994) Activity of taxol in patients with squamous cell carcinoma and adenocarcinoma of the esophagus. J Natl Cancer Inst 86:1086–1091
5. Ilson DH, Saltz L, Enzinger P, et al. (1999) Phase II trial of weekly irinotecan plus cisplatin in advanced esophageal cancer. J Clin Oncol 17:3270–3275
6. Conroy T, Etienne PL, Adenis A, et al. (2002) European Organisation for Research and Treatment of Cancer Gastrointestinal Tract Cancer Cooperative Group. Vinorelbine and cisplatin in metastatic squamous cell carcinoma of the oesophagus: response, toxicity, quality of life and survival. Ann Oncol 13:721–729
7. Hayashi K, Ando N, Watanabe H, et al. (2001) Phase II evaluation of protracted infusion of cisplatin and 5-fluorouracil in advanced squamous cell carcinoma of the esophagus: a Japan Esophageal Oncology Group (JEOG) Trial (JCOG9407). Jpn J Clin Oncol 31:419–423.
8. Trudeau ME (1996) Docetaxel: a review of its pharmacology and clinical activity. Can J Oncol 6:443–457
9. Muro K, Hamaguchi T, Ohtsu A, et al. (2004) A phase II study of single-agent docetaxel in patients with metastatic esophageal cancer. Ann Oncol 15:955–959
10. Metges J, Hennequin C, Ychou M, et al. (2001) Docetaxel as a second line chemotherapy in metastatic esophageal cancer: a French study. Proc Am Soc Clin Oncol; 20:160a (Abstr 635)
11. Sasaki Y, Shinkai T, Eguchi K, et al. (1991) Prediction of the antitumor activity of new platinum analogs based on their ex vivo pharmacodynamics as determined by bioassay, Cancer Chemother Pharmacol 27:263–270
12. Alberts DS, Fanta PT, Ruuing KL, et al. (1997) In vitro phase II comparison of the cytotoxicity of a novel platinum analog, nedaplatin (254-S), with that of cisplatin and carboplatin against fresh, human ovarian cancers. Cancer Chemother Pharmacol 39:493–497.
13. Kurata T, Tamura K, Yamamoto N (2004) Combination phase I study of nedaplatin and gemcitabine for advanced non-small-cell lung cancer. Br J Cancer 90:2092–2096.
14. Kurita H, Yamamoto E, Nozaki S, et al. (2004) Multicenter phase I trial of induction chemotherapy with docetaxel and nedaplatin for oral squamous cell carcinoma. Oral Oncol 40:1000–1006.
15. Therasse P, Arbuck SG, Eisenhauer EA, et al. (2000) New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 92:205–216
16. Osaka Y, Takagi Y, Hoshino S, et al. (2006) Combination chemotherapy with docetaxel and nedaplatin for recurrent esophageal cancer in an outpatient setting. Dis Esophagus 19:473–476
17. Abe T, et al. (2005) A phase I trial of docetaxel/nedaplatin combination therapy for advanced esophageal cancer. The 59th Annual Meeting of the Japan Esophageal Society, Tokyo, June 2005

BIOINFORMATICS ORIGINAL PAPER

Vol. 23 no. 6 2007, pages 732–738
doi:10.1093/bioinformatics/btl663

Gene expression

Genomic characterization of multiple clinical phenotypes of cancer using multivariate linear regression models

Shigeyuki Matsui[1,2,*], Masaaki Ito[3], Hiroyuki Nishiyama[3], Hajime Uno[4], Hirokazu Kotani[5], Jun Watanabe[3], Parry Guilford[6], Anthony Reeve[7], Masanori Fukushima[8,2] and Osamu Ogawa[3]

[1]Department of Pharmacoepidemiology, Graduate School of Public Health, Kyoto University, Kyoto, [2]Translational Research Informatics Center, Foundation for Biomedical Research and Innovation, Kobe, [3]Department of Urology, Kyoto University Graduate School of Medicine, Kyoto, [4]Division of Biostatistics, School of Pharmaceutical Sciences, Kitasato University, Tokyo, [5]Department of Pathology, Kyoto University Graduate School of Medicine, Kyoto, Japan, [6]Pacific Edge Biotechnology, University of Otago, Dunedin, [7]Cancer Genetics Laboratory, Department of Biochemistry, University of Otago, Dunedin, New Zealand and [8]Dvision of Clinical Trial Design and Management, Translational Research Center, Kyoto University Hospital, Kyoto, Japan

Received on October 2, 2006; revised on November 27, 2006; accepted on December 25, 2006

Advance Access publication January 18, 2007

Associate Editor: Joaquin Dopazo

ABSTRACT

Motivation: The development of gene expression microarray technology has allowed the identification of differentially expressed genes between different clinical phenotypic classes of cancer from a large pool of candidate genes. Although many class comparisons concerned only a single phenotype, simultaneous assessment of the relationship between gene expression and multiple phenotypes would be warranted to better understand the underlying biological structure.

Results: We develop a method to select genes related to multiple clinical phenotypes based on a set of multivariate linear regression models. For each gene, we perform model selection based on the doubly-adjusted R-square statistic and use the maximum of this statistic for gene selection. The method can substantially improve the power in gene selection, compared with a conventional method that uses a single model exclusively for gene selection. Application to a bladder cancer study to correlate pre-treatment gene expressions with pathological stage and grade is given. The methods would be useful for screening for genes related to multiple clinical phenotypes.

Availability: SAS and MATLAB codes are available from author upon request.

Contact: matsui@pbh.med.kyoto-u.ac.jp

1 INTRODUCTION

The development of comprehensive, gene expression microarray technology has allowed the identification of differentially expressed genes between different clinical phenotypic classes of cancer from a large pool of candidate genes. Although many of such class comparison studies assessed relation of gene expression with a single clinical phenotype, such as stage of cancer, assessment of relationship with multiple clinical phenotypes, e.g. stage and grade of cancer, would be more relevant to better understand the underlying biological structure.

In incorporating multiple phenotypic characteristics of cancer simultaneously, multivariate linear regression models or ANOVA-type models for each gene are a useful analytical tool by introducing phenotypes as covariates for the response variable of gene expression level, although many applications concern modelling of channel-specific intensities to reflect the experimental design using multichannel arrays (e.g., Kerr *et al.*, 2000, 2001; Wolfinger *et al.*, 2001; Dobbin and Simon, 2002) or oligonucleotide arrays (Chu *et al.*, 2002). In correlating gene expression data with multiple phenotypes, several groups of genes with distinct differential patterns may exist. A group of genes may relate to only a single phenotype, e.g. stage or grade, while another group of genes may relate to multiple phenotypes, e.g. both stage and grade, possibly with their interaction.

A commonly used approach for selecting genes related to multiple phenotypes is to assume a full model that incorporates all phenotypes and their possible interactions, e.g. a model with stage, grade and their interaction as covariates, to cover all possible differential patterns for multiple phenotypes. Then, an overall test for the null hypothesis of no effect of all the covariates on gene expression is performed with some control for false positives (Chen *et al.*, 2005; Matsui, 2006). A drawback to this approach is that, although the overall test could detect genes with any differential patterns, the power of this test may be smaller for genes for which reduced models are correct. One may want to detect genes with various differential models equally. Only a single model should not be fitted for all genes exclusively, unless a particular differential pattern is of interest.

*To whom correspondence should be addressed.

One idea to improve the power for various differential models would be to invoke some model selection. Model selection may also be warranted to gain additional insights on selected genes. For example, a group of genes for which the same model is selected have the potential to relate to the same aspect of disease biology. One approach is to perform a structural hypothesis testing. For example, we first assume the full model with stage, grade and their interaction and test if there is an interaction effect between stage and grade for all genes. For those genes where an interaction exists, the full model can be selected. For those where there is not, the reduced model with just stage and grade can be fit and the main effects of stage and grade are tested separately. A critical issue of this approach, however, is how to determine the significance levels for these tests. One possible approach is to determine the significance levels based on a criterion to control an error rate on false positives. However, there could be multiple combinations of significant levels for main and interaction effects for a given level of an overall error rate. Apart from technical issues, one may also argue in the first place that model selection is a different problem of gene selection and hence another criterion should be used for model selection.

In this article, we develop a method to select genes related to multiple clinical phenotypes via model selection based on a set of candidate, multivariate linear regression models. For each gene, all candidate models are fit and a model is selected based on a criterion of prediction error. Specifically we select the model for which the doubly-adjusted R-square proposed by Okuno *et al.* (1977) is maximized. This model selection procedure can be expressed as model selection based on hypothesis testing described earlier, but the significance levels are determined from the perspective of prediction error (see Section 2). Also, this model selection criterion is approximately equal to popular criteria based on Mallow's Cp (Mallows, 1973) and AIC (Akaike, 1973). Meanwhile, the doubly-adjusted R-square is a reasonable statistic for gene selection because of its link to an overall test for the null hypothesis of no effect of all phenotypic variables on gene expression. Procedures to control the multiplicity from examining the large number of genes can be developed. A simulation study is conducted to evaluate the performance of the proposed method. Lastly, we illustrate the methods using a bladder cancer data set to correlate pre-treatment gene expression levels with pathological stage and grade.

2 METHODS

2.1 Multivariate linear regression models

Assume values $Y_{j,i}$ for $j=1,\ldots,J$ genes from sample $i=1,\ldots,n$. For cDNA arrays these are typically normalized log-ratios and for oligonucleotide arrays they are normalized log signals. For each gene, the expression data is linked to multiple phenotypes via multivariate linear regression models. We suppose that there are, in total, p variables, $x_1,\ldots,x_p$, for which their association with gene expression is of interest. Note that the set of variables may include those representing interaction effects of different phenotypes, for example, an interaction of two different phenotypes, $x_{k1}x_{k2}$, where x_{k1} and x_{k2} represent the respective phenotypes ($k_1 \neq k_2$). We assume various candidate models using the set of all the p variables and subsets of it as covariates. When k covariates ($1 \leq k \leq p$) are linked to the expression level for gene j, we assume the (candidate) linear model,

$$Y_{j,i} = \beta_0 + \beta_1 x_{i1} + \cdots + \beta_k x_{ik} + \varepsilon_{j,i} \quad (1)$$

where $\beta_0, \beta_1, \ldots, \beta_k$ are regression coefficients. The error term, $\varepsilon_{j,i}$, is assumed independently and identically distributed the normal distribution $N(0, \sigma_j^2)$.

2.2 The Doubly-adjusted R-square

In this section, we restrict our attention to analysis of a particular gene and subscript j is dropped for brevity. The doubly-adjusted R-square is related to the prediction sum of squares (PRESS) (Hocking, 1976), defined by $\text{PRESS} = \sum_{i=1}^{n} (y_i - \hat{y}_{(i)})^2$, where $\hat{y}_{(i)}$ is the predicted value of y_i obtained after omitting sample i from the fitting process ($i=1,\ldots,n$). The PRESS is a statistic related to prediction error. If the model is correctly specified, the expected value of PRESS reduces to $(n+k+1)\sigma^2$. Using the commonly used, unbiased estimator for the parameter σ^2, $RSS/(n-k-1)$, the expected PRESS can be estimated by $(n+k+1)RSS/(n-k-1)$.

The doubly-adjusted R-square is defined by one minus the ratio of estimated expected prediction errors, the expected prediction error under the linear model with k covariates and that under the linear model with no covariate ($k=0$) (Okuno *et al.*, 1977),

$$R^2_{DA} = 1 - \frac{[(n+k+1)/(n-k-1)]RSS}{[(n+1)/(n-1)]TSS},$$

or

$$R^2_{DA} = 1 - \frac{n+k+1}{n+1}\frac{n-1}{n-k-1}(1-R^2) \quad (2)$$

where

$$R^2 = 1 - \frac{RSS}{TSS} \quad (3)$$

denotes the coefficient of multiple determination or the R-square statistic. Here $TSS = \sum_{i=1}^{n} (y_i - \bar{y})^2$ is the total sum of squares and $RSS = \sum_{i=1}^{n} (y_i - \hat{y}_i)^2$ is the residual sum of squares, where $\hat{y}_i$ is the fitted value for y_i and $\bar{y} = \sum_{i=1}^{n} y_i/n$ is the average of gene expression across all samples. For R^2_{DA}, the expected PRESS is used, instead of the usual sum of squares such as RSS and TSS, which are used for R^2 in (3).

The statistic R^2_{DA} has a one-to-one correspondence with an F-statistic for the overall test of the null assumption that all of the k regression coefficients are zero. Note that this null assumption represents no effects of all the p variables because no effects of the other $p-k$ variables are assumed in model (1). The F-statistic is given by

$$F = \frac{(TSS - RSS)/k}{RSS/(n-k-1)} \quad (4)$$

which has the relation with R^2,

$$R^2 = \frac{kF}{kF+n-k-1} \quad (5)$$

As such, R^2_{DA} has correspondence with the F-statistic through (2).

For each gene, we select the model for which R^2_{DA} is maximized over candidate models. Consider comparison of the two models, one with k covariates and the other with $(k+1)$ covariates after the entry of one additional covariate into the model with k covariates. The model with $(k+1)$ covariates will be selected if $R^2_{DA,(k+1)} \geq R^2_{DA,(k)}$.

Another popular criterion for model selection is based on the partial F-statistic, which can be expressed as

$$F_{(k)} = \frac{R^2_{(k+1)} - R^2_{(k)}}{(1 - R^2_{(k+1)})/(n-k-2)}$$

The criterion $R^2_{DA,(k+1)} \geq R^2_{DA,(k)}$ corresponds to

$$F_{(k)} \geq \frac{2n}{n+k+1} \approx 2$$

when $n \gg k$ (Okuno *et al.*, 1977). Another popular criterion is based on Mallow's Cp (Mallows, 1973),

$$Cp = \frac{RSS}{\sigma^2} + 2(k+1) - n$$

for the model with k covariates. The AIC (Akaike, 1973) is also a popular statistic for model selection, although assumption for the error term distribution is needed. For normally distributed error terms with known variance, AIC can be expressed as

$$AIC = \frac{RSS}{\sigma^2} + 2(k+1) + \text{const.}$$

for the model with k covariates, which is identical with Cp. The model with $(k+1)$ covariates may be selected if $Cp_{(k+1)} \leq Cp_{(k)}$ or $AIC_{(k+1)} \leq AIC_{(k)}$. When the unbiased estimate for σ^2 under the model with $(k+1)$ covariates, $RSS_{(k+1)}/(n-k-2)$, is replaced with σ^2, these criteria may correspond to

$$F_{(k)} \geq 2.$$

As such, the criterion based on R^2_{DA} is approximately equal to the criteria based on Cp or AIC.

2.3 Selection of model and gene

We invoke model selection for each gene before gene selection. For each gene, we perform model selection based on R^2_{DA}, i.e. selecting the model for which is maximized. Then, the maximum R^2_{DA} is used as the statistic for gene selection, and genes with the greatest values of the maximum R^2_{DA} are selected. A rationale for using R^2_{DA} for gene selection is related to its correspondence with the overall F-test of no relations between all the p variables and gene expression as noted in the previous section. For genes for which this null assumption is incorrect, R^2_{DA} for some candidate models or the maximum of R^2_{DA} across candidate models may tend to have larger values, compared with genes for which the null assumption is correct. This tendency may be true for genes with any differential pattern for gene expression as long as this is captured by one of candidate models. Thus, our procedure is to select genes related to multiple phenotypes irrespective of their differential patterns via the optimization in gene selection. Note that other model selection criteria such as Cp and AIC may not have the correspondence with the overall test because of standardization across candidate models for each gene, which would be a drawback for using these statistics for gene selection. For example, if all p variables are used as covariates in estimating σ^2 for each gene, Cp for the model with k covariates may reduce to $2k-p+1$ for all genes.

The multiplicity issue by repeated examinations across genes can be adjusted by controlling the family-wise error rate (FWER) or the false discovery rate (FDR), where the FWER is the probability of making one or more false positives and the FDR is the expected proportion of false positives among the positives declared (Dudoit *et al.*, 2003). Note that the control of these error rates does not relate to individual effects but to the overall test of the null assumption of no effects for all the p variables. Many authors now favour the FDR over the FWER as the appropriate error measure for exploratory microarray studies because the FWER approach can be very conservative. A practical and conceptually simple approach to providing procedures to control the FDR conservatively is to fix the rejection region beforehand and to estimate the FDR (Storey, 2002). A gene is declared to be significant if the maximum R^2_{DA} for the gene is equal to or greater than a cut-off point on this statistic. For a given cut-off point, we can estimate the FDR conservatively by a multivariate permutation procedure with random permutations of the assignment of the set of all p variables to derive the null distribution of the maximum R^2_{DA}, from which the average of (false) positives can be calculated as an estimate of the expected false positives. For controlling FWER, the null distribution of the maximum of the maximum R^2_{DA} across genes is derived from the random permutations, which is referred to the observed value of the maximum R^2_{DA} to obtain an adjusted P-value.

3 RESULTS

3.1 Simulation

We assessed adequacy of the proposed method through a small simulation study. Particularly we compared the power of our method with that of the conventional method that uses a single model with all p variables as covariates exclusively and select genes based on the overall F-test for this model. We considered three distinct phenotypes. The state of each phenotype was dichotomized, and let x_1, x_2 and x_3 be binary variables representing respective phenotypes. Let $x_h = 1$ for a particular state of phenotype and $x_h = 0$ for the other states ($h = 1, 2, 3$). Thus, there are eight combinations in total for the level of the three phenotypes, $(x_1, x_2, x_3) = (0,0,0)$, $(0,0,1)$, $(0,1,0)$, $(0,1,1)$, $(1,0,0)$, $(1,0,1)$, $(1,1,0)$ and $(1,1,1)$. We considered six samples for each combination (the total number of samples was 48).

We assessed the power for selecting a single informative gene which is related to phenotypes. We considered 1000 genes and, of the 1000 genes, 1 gene was informative and the other 999 were non-informative (0.1% of the whole gene is informative). For the informative gene, we assumed the model $\{1\}$, $\{1,2\}$, $\{1,2,1\times2\}$, $\{1,2,3\}$ or $\{1,2,3,1\times2,1\times3,2\times3,1\times2\times3\}$. For example, the model $\{1,2,1\times2\}$ is to a linear model with the three covariates, x_1, x_2 and x_1x_2. The error term ($\varepsilon_{j,i}$ in(1)) is assumed independently and identically distributed the standard normal distribution for all genes. The size of regression coefficients ranged from 0.0 to 2.0. For the informative gene with the model, $\{1,2,1\times2\}$, we assumed the differential patterns where the samples with one of the four combinations for the level of the two binary phenotypic variables, x_1 and x_2, e.g. samples with $(x_1, x_2) = (0,0)$, irrespective of the level of x_3, had higher expressions compared with the other samples. Similarly, for the informative gene with the model, $\{1,2,3,1\times2,1\times3,2\times3,1\times2\times3\}$, we assumed the differential patterns where the samples with one of the eight combinations for the level of the three binary phenotypic variables, x_1, x_2 and x_3, e.g. samples with $(x_1, x_2, x_3) = (0,0,0)$, had higher expressions compared with the other samples.

For each simulation configuration, we performed the proposed method and the conventional method. For the proposed method, we introduced the four variables representing interaction effects, x_1x_2, x_1x_3, x_2x_3 and $x_1x_2x_3$, in addition to the three variables, x_1, x_2 and x_3, as covariates. For the seven covariates, we assumed the following 12 candidate models, $\{1\}$, $\{2\}$, $\{3\}$, $\{1,2\}$, $\{1,3\}$, $\{2,3\}$, $\{1,2,1\times2\}$,