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RESEARCH ARTICLE

The effect of IGF-I receptor blockade for human esophageal squamous cell carcinoma and adenocarcinoma

Yasushi Adachi · Hirokazu Ohashi · Arisa Imsumran · Hiroyuki Yamamoto · Yasutaka Matsunaga · Hiroaki Taniguchi · Katsuhiko Nosho · Hiromu Suzuki · Yasushi Sasaki · Yoshiaki Arimura · David P Carbone · Kohzoh Imai · Yasuhisa Shinomura

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Abstract Insulin-like growth factor-I receptor (IGF-IR) signaling is required for carcinogenicity and tumor development, and this pathway has not been well studied in human esophageal carcinomas. Esophageal cancer is one of the human cancers with the worst prognosis and has two main histologies: squamous cell carcinomas (ESCC) and adenocarcinoma (EAC). Previously, we have reported that detection of the IGF axis may be useful for the prediction of recurrence and poor prognosis of ESCC. We have also shown the successful therapy for several gastrointestinal cancers using recombinant adenoviruses expressing dominant negative IGF-IR (ad-IGF-IR/dn). The aim of this study is to develop potential targeted therapeutics to IGF-IR and to assess the effect of IGF-IR blockade in both of these types of esophageal cancer. We determined immunohistochemical expression of IGF-IR in a tissue microarray. We then assessed the effect of IGF-IR blockade on signal transduction, proliferation, apoptosis, and motility. Ad-IGF-IR/dn, a tyrosine kinase inhibitor, BMS-536924, and adenovirus expressing shRNA for IGF-IR were used. IGF-IR expression was common in both tumor types but not in normal tissues. IGF-IR was detected in metastatic sites at similar levels compared to the primary site. IGF-IR inhibition suppressed proliferation and colony formation in both cancers. IGF-IR blockades up-regulated both stress- and chemotherapy-induced apoptosis and reduced migration. Although IGF-IR/dn blocked ligand-induced activation of Akt-1 mainly, BMS-536924 effectively blocked both activation of Akt and MAPK. The IGF axis might play a key role in tumor progression of esophageal carcinomas. The IGF-IR targeting strategies might thus be useful anticancer therapeutics for human esophageal malignancies.

Keywords Dominant negative · EAC · ESCC · IGF-IR · TKI

Abbreviations

ad-IGF-IR/482st	Adenovirus expressing IGF-IR /482st
ad-IGF-IR/950st	Adenovirus expressing IGF-IR/950st
ad-shIG F-IR	Adenovirus expressing short-hairpin IGF-IR
des(1–3)IGF-I	NH_2 terminally truncated IGF-I
dn	Dominant negative
EAC	Esophageal adenocarcinoma
ESCC	Esophageal squamous cell carcinoma
ERK	Extracellular signal-regulated kinase
IGF	Insulin-like growth factor
IGFBP	IGF binding protein
IGF-IR	IGF-I receptor
IGF-IR/482st	Truncated IGF-IR of 482 amino acid long

Yasushi Adachi, Hirokazu Ohashi, and Arisa Imsumran contributed equally to this work.

Y. Adachi (✉) · H. Ohashi · A. Imsumran · H. Yamamoto · Y. Matsunaga · K. Nosho · H. Suzuki · Y. Sasaki · Y. Arimura · K. Imai · Y. Shinomura
First Department of Internal Medicine, Sapporo Medical University, S-1, W-16, Chuo-ku, Sapporo 060-8543, Japan
e-mail: yadachi@sapmed.ac.jp

H. Taniguchi · K. Imai
The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

D. P. Carbone
James Thoracic Center, The Ohio State University, Columbus, OH, USA

IGF-IR/950st	Truncated IGF-IR of 950 amino acid long
IGF-IR/dn	Dominant negative form of IGF-IR
InsR	Insulin receptor
mAb	Monoclonal antibody
PI3-K	Phosphatidylinositide 3-kinase
TKI	Tyrosine kinase inhibitor

Introduction

Esophageal cancer is one of the cancers with the worse prognosis worldwide [1]. At the time of diagnosis, more than half of patients have either unresectable tumors or metastatic ones. Even after a curative-intent surgical operation, the 5-year survival is still limited [2], and the therapy for unresectable esophageal carcinomas is typically minimally effective. Therefore, we must aim to seek new therapeutic options for this disease. The main types of human esophageal tumor are squamous cell carcinoma (ESCC) and adenocarcinoma (EAC).

Recently, advances in molecular research have brought new therapeutic strategies, including small molecule tyrosine kinase inhibitors (TKI) and monoclonal antibodies (mAb), into clinical testing. One group of new targets is the tyrosine kinase receptors. The insulin-like growth factor (IGF) family is a promising candidate [3, 4]. Agents targeting the IGF-I receptor (IGF-IR) pathway are moving into the clinic. Toward that end, we have studied this pathway in esophageal cancers.

IGF-IR is a heterotetramer of two α- and two β-chains [5]. Binding of the ligands IGF-I and IGF-II to IGF-IR causes receptor autophosphorylation and activates multiple signaling pathways, including ras/extracellular signal-regulated kinase (ERK) and the phosphatidylinositide 3-kinase (PI3-K)/Akt-1 axes [6]. Activation of IGF-IR is regulated by multiple factors, including IGF binding proteins (IGFBP) and IGF-2 receptor [7–9]. Elevation of serum IGF-I increases the risk of developing several cancers [10], and IGF-IR is essential for both malignant transformation and progression [3, 4]. Reduction of IGF-IR can induce apoptosis in tumors but produces only growth slowing in untransformed cells, suggesting that it might be an excellent target for therapeutic intervention [3]. IGF-IR knockout mice are viable (though physically small), indicating that relatively normal development and differentiation can occur in its absence [11]. These findings suggest a potential basis for tumor selectivity in therapeutic applications.

Human esophageal epithelial cells express IGF-IR, and IGF-I can stimulate both DNA synthesis and proliferation in these cells [12–14]. Salivary IGF-I continuously bathes the esophageal lumen and is in a free form (not bound to IGFBP, unlike the serum pool), which could enhance its binding ability to receptors on the esophageal mucosal cells [15]. These data indicate that the IGF/receptor may play important roles in homeostasis and esophageal premalignancy [14].

Both IGF-IR and IGFs are overexpressed in esophageal cancer tissues compared to normal ones [16–18]. In addition, IGFBP3 and an IGF-IR antibody suppress cancer cell proliferation [19, 20]. However, the role of the IGF axis in esophageal cancer has not been adequately studied. We reported previously that expression of IGF-IR and IGF-II were detected in 60 and 50% of ESCC, respectively, and were associated with invasion depth, metastasis, advanced tumor stage, and recurrence [21]. Patients with ESCC expressing both IGF-IR and IGF-II had a significantly shorter survival rate than those expressing either alone or neither in both single and multivariate analysis. Dominant negative for IGF-IR (IGF-IR/dn) suppressed proliferation and up-regulating chemotherapy-induced apoptosis through blocking ligand-induced Akt activation in an ESCC cell line, TE-1 [21].

In addition, there is a strong positive association between visceral obesity (metabolic syndrome) and risk of EAC, and the IGF axis is speculated to relate to both obesity and EAC [22]. IGF-IR expression in resected EAC was significantly higher in viscerally obese patients than in those of normal weight. Disease-specific survival was longer in patients with IGF-IR-negative EAC than in those with IGF-IR-positive tumors [23]. Thus, there are several lines of evidence that the IGF axis may play an important role in EAC.

There are several possible approaches to blocking IGF-IR signaling with therapeutic intent [24], including blocking the ligand or receptor using mAbs [25, 26] or TKIs [27, 28]. All of these are complicated by the high homology of this receptor to the insulin receptor (InsR). An approach that is intrinsically specific for IGF-IR is to use dominant negative or soluble IGF-IR receptor approaches to specifically inhibit the function of the wild-type receptor [29, 30]. We have constructed two different adenoviruses expressing IGF-IR/dn (ad-IGF-IR/dn) [31–34]. Ad-IGF-IR/482st encodes a truncated extracellular domain of IGF-IR (without the transmembrane domain) and thus produces a secreted protein that affects neighboring cells in addition to the transduced cells (a bystander effect). Another ad-IGF-IR/950st encodes a receptor that lacks the tyrosine kinase domain and thus remains on the membrane of the transduced cells to form non-functional receptor complexes. We have reported that ad-IGF-IR/dn may be a useful therapeutic strategy against several gastrointestinal tumors [21, 31, 32, 34, 35]. We have also reported that the adenoviral vector-based approach to express a short-hairpin inhibitory RNA of IGF-IR (ad-shIGF-IR) induced effective IGF-IR silencing in gastrointestinal cancers as manifested by effective blockade of the downstream pathway of IGF-IR and antitumor effects [36]. A dual targeting TKI for IGF-IR/InsR, BMS-536924, may have an advantage compared to a single targeting TKI,

as transformed cells can also use insulin receptor activation of similar signaling pathways for proliferation in addition to IGFR signals [35, 37].

In order to evaluate the expression of IGF-IR in EAC and in metastatic sites of ESCC, we analyzed an esophageal cancer tissue microarray immunohistochemically. To assess IGF-IR blockade for both esophageal cancers, histologies ESCC and EAC, we used several strategies including IGF-IR/dns, shIGF-IR, and BMS-536924.

Methods

Materials, cell lines, and recombinant adenovirus vectors

Anti-Akt1(c-20), anti-ERK1(K-23), anti-phospho-ERK1(E-4), ant-IGF-I(G-17), and anti-IGF-IRβ(2C8) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-phospho-Akt(Ser473) was from Cell-Signaling Technology (Beverly, MA, USA). Anti-IGF-IR(Ab-4) was from Oncogene Research Products (Cambridge, MA, USA) and anti-IGF-II was from Peninsula Laboratories (San Carlos, CA, USA). PI3-K inhibitors, wortmannin and LY294002, p38-MAPK inhibitor SB203580, cisplatin (CDDP), and 5-fluorouracil (5-FU) were purchased from Sigma (St. Louis, MO, USA), and MEK1 inhibitor PD98059 was from Cell Signaling. Recombinant human IGF-I and IGF-II were purchased from R&D systems (Minneapolis, MN, USA) and des(1–3)IGF-I from GroPep (Adelaide, Australia). All human esophageal cancer cell lines (Fig. 1) were obtained from the Japanese Cancer Collection of Research Bioresources Cell Bank (Tokyo, Japan), Riken Bioresource Center Cell Bank (Tsukuba, Japan), and European Collection of Cell Cultures (Salisbury, UK). Cells were passaged in RPMI1640 and DMEM, both with 10% fetal bovine serum.

Recombinant adenoviruses expressing IGF-IR/dn (482 and 950 amino acids long, IGF-IR/482st and IGF-IR/950st, Ad-IGF-IR/482st and Ad-IGF-IR/950st, respectively) were generated as described previously by homologous recombination [31]. Recombinant adenovirus vectors expressing shIGF-IR (ad-shIGF-IR) were generated as described previously [38]. An adenovirus expressing β-galactosidase was used as a control (ad-LacZ). Scrambled shRNA adenovirus (ad-Scr) is another control that has a short hairpin sequence but no specific target, also as described previously.

BMS-536924 was kindly provided by Bristol-Myers Squibb (New York, NY, USA). Stock solution was prepared in DMSO and stored at −20°C.

Immunohistochemical analysis

The paraffin-embedded esophageal tissue microarray (ES208) was purchased from US Biomax (Rockville, MD, USA). After deparaffinization, endogenous peroxidase activity was blocked. Antibodies were applied after blocking with normal goat serum. Sections were incubated with the anti-rabbit secondary antibody (Santa Cruz Biotechnology) and a strep tavidin-HRP followed by exposure to the diaminobenzidine tetrahydrochloride substrate (Dako). The sections were counterstained in Mayer's hematoxylin and mounted. Immunostaining signals were scored by two independent observers. Semiquantitative scores were given as the score of the percentage of positive cells plus the score of the staining intensity. The scoring criteria of the percentage of positive cells were as follows: score 0, 0–5% positive cancer cells; score 1, 6–25%; score 2, 26–50%; score 3, 51–75%; score 4, 76–100% positive. The intensity score was given as follows: score 0, no staining; score 1, weak/equivocal; score 2, moderate; score 3,

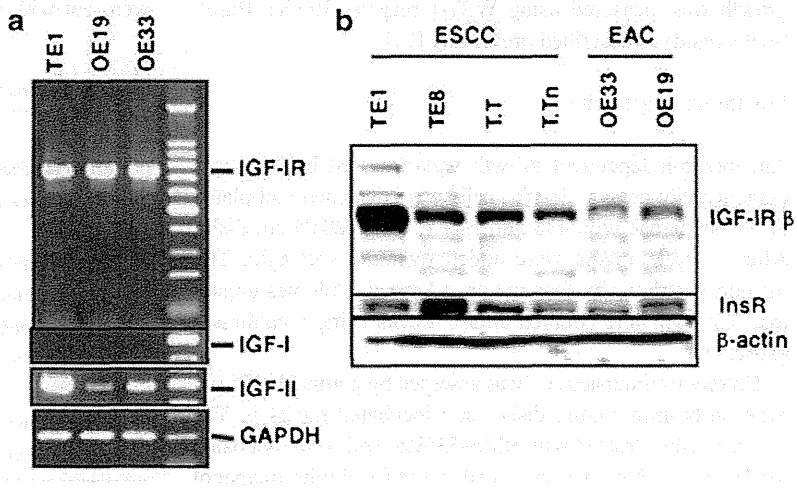


Fig. 1 The expressions of IGF-axis in esophageal carcinoma cell lines. **a** RT-PCR revealed that three cells express mRNAs of IGF-II and IGF-IR but not IGF-I. **b** Western blotting showed that two EAC and four ESCC cells express both IGF-IR and InsR

strong staining. The final scores were from 0 to 7 and four or more were considered positive.

Reverse transcription PCR

Total RNA from cells was isolated by the acid guanidinium thiocyanate–phenol–chloroform method. Primer sets for the amplification of IGF-I cDNA sequences were 5′-CACTGT CACTGCTAAATTCA-3′ and 5′-CTGTGGGCTTGTTGAAA TAA-3′ [39]. Primers for IGF-II cDNA were 5′-AGTCGATGC TGGTGCTTCTCA-3′ and 5′-GTGGGCGGGGTCTTGG GTGGGTAG-3′ [40]. Primers for IGF-IR were 5′-ATTGAG GAGGTCACAGAGAAC-3′ and 5′-TTCATATCCTGTTTT GGCCTG-3′ [40]. Randomly primed cDNAs were prepared from 1 mg of total RNA by M-MLV reverse transcriptase (Takara, Japan) and amplified by PCR. For amplification of these sequences, 35 cycles of PCR was programmed as follows: 94°C, 30 s; 60°C, 30 s; 72°C, 30 s.

Western blotting

Cells were cultured in serum-free medium for 24 h and then stimulated with 20 ng/ml IGF-I or 10 nM insulin. Cell lysates were prepared as described previously [31]. Equal aliquots of lysate (100 μg) were separated by 4–20% SDS-PAGE and immunoblotted onto polyvinylidene Hybond-P membrane (Amersham, Arlington Heights, IL, USA). Analysis was performed using the indicated antibodies, and bands were visualized by ECL (Amersham).

Assessment of the effect on in vitro cell growth

Tumor cells were grown to 70% confluence in six-well plates and infected with adenovirus. The number of cells was then assayed by Trypan blue staining.

Four thousand cells were seeded into the wells of a 96-well plate, and each was infected with adenovirus or control. Cell growth was measured using WST-1 reagent (Roche, Basel, Switzerland) as described previously [21].

In vitro tumorigenicity

Anchorage-independent growth was assessed by soft agar clonogenicity assays. Briefly, cells were detached and plated in 0.2% agarose with 1% underlay (2×10^4 cells/5-cm dish). After 1 week, media were added over the soft agar. The medium overlay was changed after 1 week. Colonies greater than 125 μm were counted after 3 weeks using a calibrated graticule.

Colony forming activity was assessed by plating 3×10^3 per plate on 60-mm culture dishes and incubated for 24 h. The cells were then treated with BMS-536924 and were incubated for 14 days. After air-drying, cells were fixed with methanol and stained with Giemsa solution. Colonies containing 50 cells or more were counted.

Measurement of apoptosis

The DNA fragmentation assay was performed as follows: low molecular weight DNA was extracted with 0.5% Triton X-100, 10 nM EDTA, and 10 mM Tris–HCl, pH 7.4, treated with 400 μg/ml RNase A and then proteinase K for 1 h at 37°C, ethanol-precipitated, and subjected to 1% agarose gel electrophoresis. The gels were stained with 1 μg/ml ethidium bromide. Early apoptosis was quantified by staining with Annexin-V-FITC according to the manufacturer's protocol (BD Biosciences) and measured by flow cytometry. Cells undergoing apoptosis showed an increase in Annexin-V binding but excluded propidium iodide. TUNEL assays were performed with in situ apoptosis detection kit (Takara) following the manufacturer's protocol. Caspase-3 colorimetric protease assay was performed following the manufacturer's protocol (Caspase-3 Colorimetric Protease Assay Kit; MBL). In brief, 3×10^6 cells were lysed in 100 μl of chilled cell lysis buffer, and total cell lysates (100 μg) were incubated with 4 mM VETD-pNA Substrate (200 μM final concentration) at 37°C for 1 h. Caspase-3 activity was measured by colorimetric reaction at 405 nm.

First, cancer cells infected with Ad-IGF-IR/dns or Ad-LacZ were induced with 10 mJ/cm^2 UV light. To assess the efficacy of IGF-IR/dn on chemotherapy-induced apoptosis, tumor cells were treated for 24 h with 1 mM 5-FU or 50 μM cisplatin.

Migration assay

Wounding assays were performed using a modification of the procedure described by Pennisi et al. [41]. Briefly, six-well chambers were prepared by scratching registration marks onto the slide surface. TE1 cells (infected with adenoviruses) were plated, grown normally for 48 h, and starved overnight. Cells were cut with a cell scraper, and five images were captured

Table 1 Summary of immunohistochemical expression of IGF-IR

	IGF-IR (+)		
Normal esophageal mucosa	0/7	0%	
Esophageal carcinoma	31/57	54%	$p = 0.0111$ (Fisher)
	IGF-IR (+)		
Squamous cell carcinoma	23/34	68%	
Primary sites	15/23	65%	
Metastasized sites	8/11	73%	
Lymph node	6/9	67%	
Skin	2/2	100%	
Adenocarcinoma	8/22	36%	
Adenosquamous carcinoma	0/1	0%	

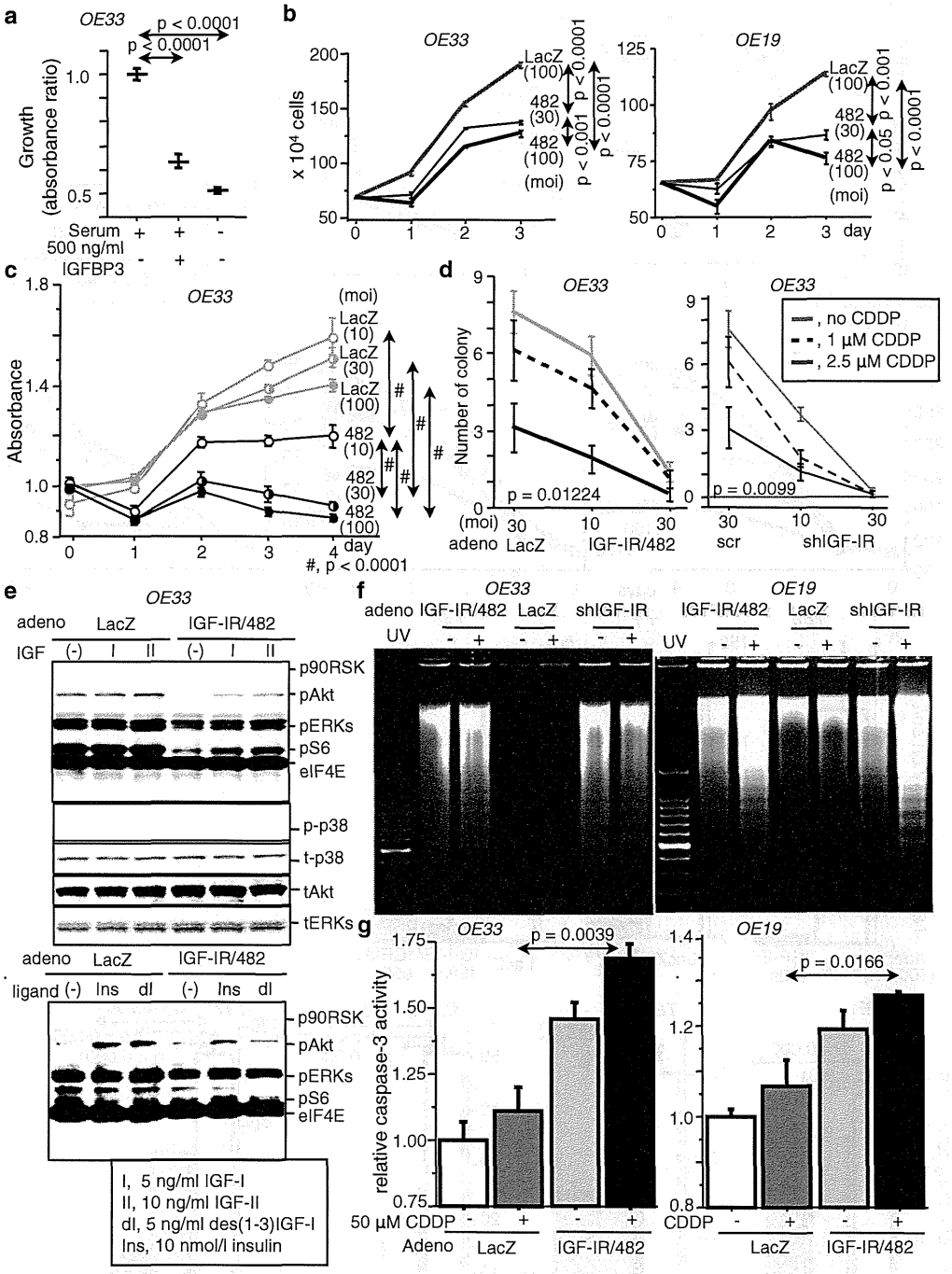


Fig. 2 The effect of IGF-IR on EAC cells. **a** WST-1 assay showed cell growth of OE33, 48 h of culture with/without IGFBP3. **b** Trypan blue assay showed the number of viable cells. **c** WST-1 assay revealed cell proliferation of adenoviruses-infected OE33. **d** Colony formation assays showed the effect of IGF-IR/dn and cisplatin on colony formation. **e** OE33 was stimulated for 5 min with ligands in serum-free medium. Western blotting showed signal transduction. **f** DNA fragmentation assay detected UV-induced apoptosis. **g** Cells were treated for 24 h with cisplatin. Then, caspase-3 assays were performed

along the cut surface on an Olympus IX-71S1F-2 microscope (Tokyo, Japan) using a ×20 objective. Additional images were captured 24 h later. For each experiment, the number of migrating cells was counted by two independent observers [41].

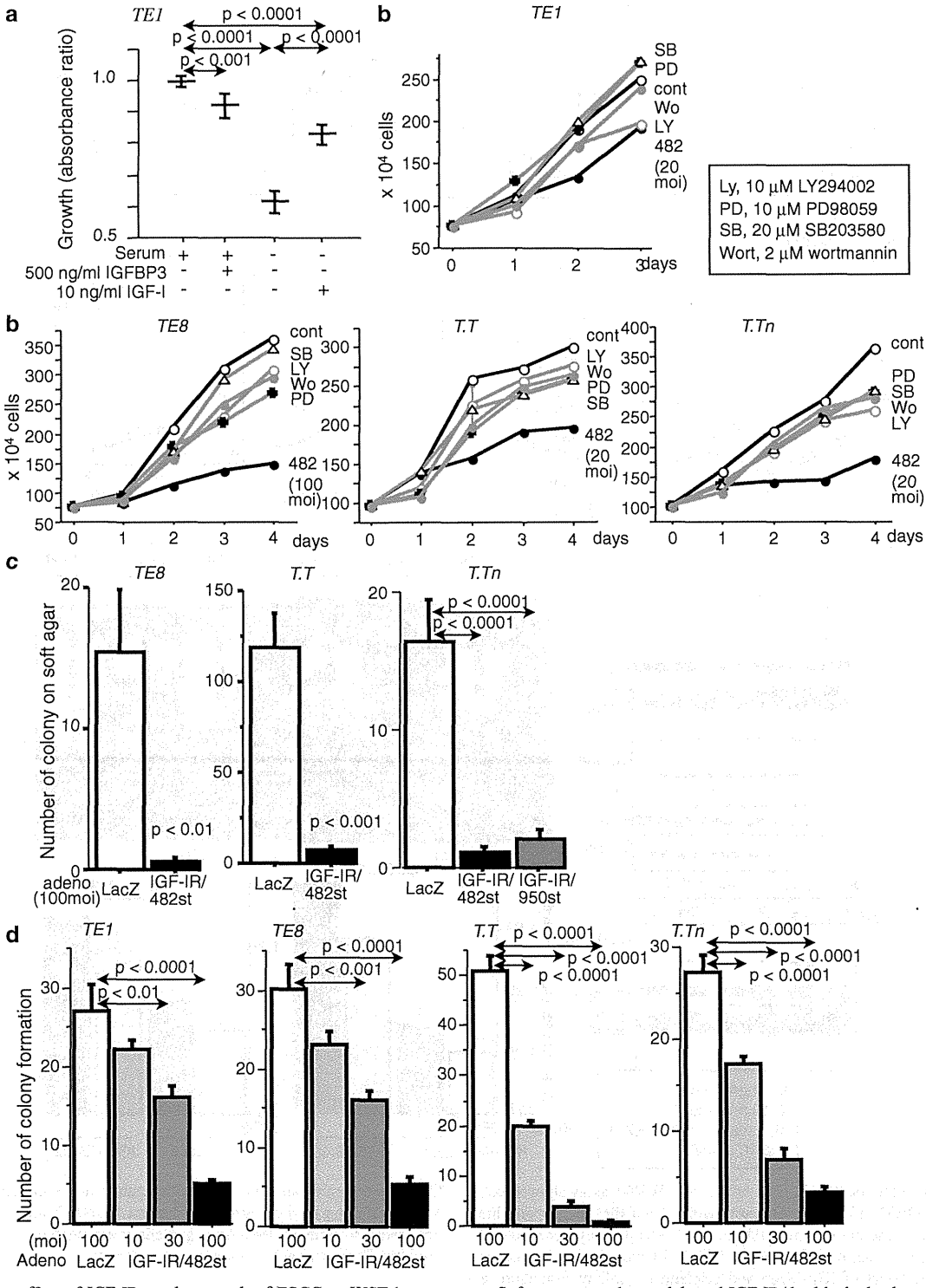


Fig. 3 The effect of IGF-IR on the growth of ESCC. **a** WST-1 assay showed cell growth after 48 h of culture. **b** Trypan blue assay showed the viable cell number of ESCC cells with several inhibitors or IGF-IR/482st. **c** Soft agar assays detected that ad-IGF-IR/dns blocked colony formation. **d** Colony formation assay showed the effect of IGF-IR/482st on colony number

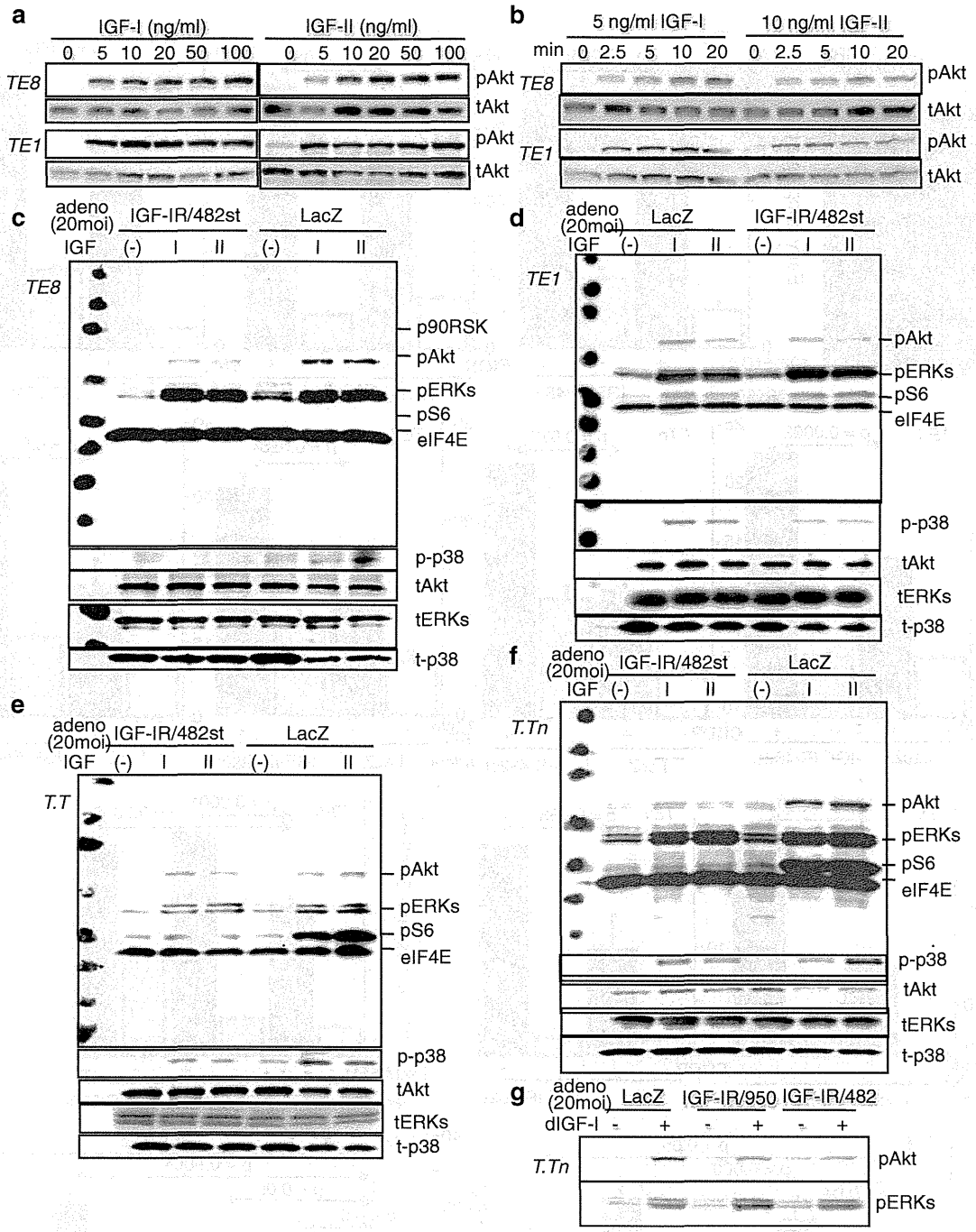
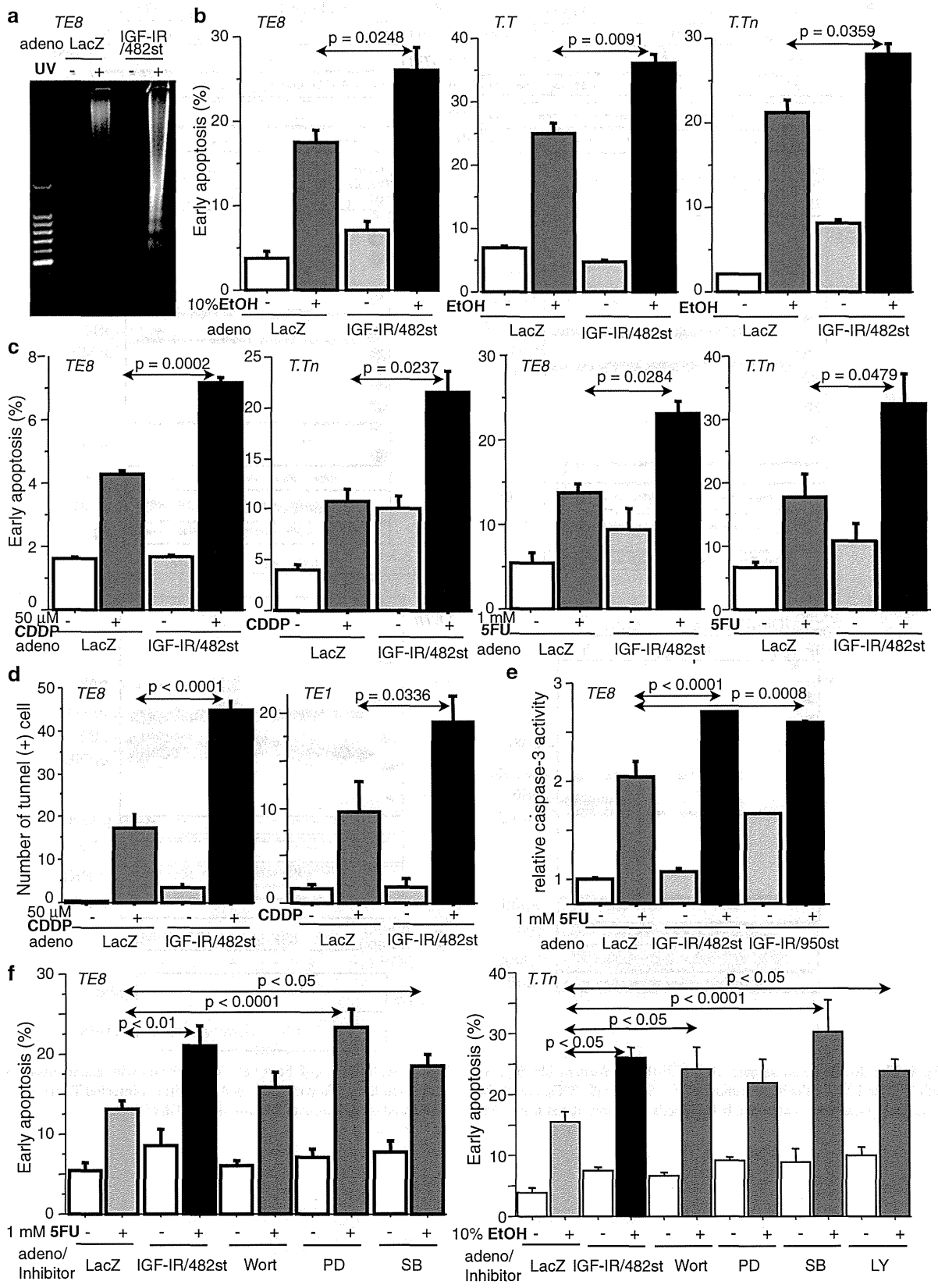


Fig. 4 The down-stream signals from IGF-IR by Western blotting. **a** Both TE8 and TE1 cells were stimulated for 5 min with IGFs, and then whole cell lysates were extracted. **b** Both cells were stimulated from 0 to 20 min with IGFs. **c–f** Four cell lines infected with adenoviruses were stimulated for 5 min with IGFs. **g** Adenoviruses-infected T.Tn cells were stimulated for 5 min with 5 ng/ml des(1–3)IGF-I



◀ **Fig. 5** Apoptotic induction in ESCC. **a** DNA fragmentation assay showed UV-induced apoptosis. **b** Annexin-V assay revealed ethanol (EtOH)-induced early apoptosis. **c** Cells were treated with chemotherapy for 24 h. Then, annexin-V assay detected early apoptosis. **d** TUNEL assay revealed cisplatin-induced apoptosis. **e** Caspase-3 assays demonstrated 5-FU-induced apoptosis. **f** Annexin-V assay detected early apoptosis in ESCC cells with several inhibitors or IGF-IR/dn

Statistical analysis

Statistical significance of difference between IGF-IR expressions was determined by Fisher's exact probability test.

The results of in vitro experiments are presented as means ± SE for each sample. The statistical significance of difference was determined by one-way ANOVA or two-factor factorial ANOVA. P values less than 0.05 were considered to indicate statistical significance.

Results

The expressions of IGF axis in esophageal cancers

In the previous paper, we reported that many ESCC cell lines express both IGF-IR and IGF-II, but a few cells express IGF-I [21]. We evaluated the mRNA expression of both IGF-IR and its ligands in two esophageal adenocarcinoma cell lines using RT-PCR (Fig. 1a). Like the control ESCC, TE1, both IGF-IR and IGF-II messages were identified. However, none expressed IGF-I mRNA. Then, we assessed the protein expressions of both IGF-IR and InsR using Western blotting (Fig. 1b). Both receptors were expressed in the two adenocarcinoma cell lines, and those expression levels were less than those of four ESCC lines.

Tissue array data showed that IGF-IR was expressed in cancer tissue more frequently than the normal mucosa (54 and 0%, respectively, $p = 0.0111$; Table 1). The expression of IGF-IR tended to be lower in EAC compared to ESCC (eight out of 22 primary EAC and 15 of 23 primary EACC). In ESCC, the IGF-IR expression ratio of metastatic sites tended to be higher, but not significantly so than that of the primary sites (73 and 65%, respectively).

These results indicate that both ligands and receptors are expressed in many esophageal carcinomas, implying that the IGF/IGF-IR axis might play some role in not only ESCC but also EAC.

The effect of IGF-IR blockade on EAC cell lines

The natural inhibitor of IGFs, IGFBP3, suppressed the growth of OE33 to a similar level as that observed when they are cultured in serum-free media (Fig. 2a). Ad-IGF-IR/dn could reduce in vitro cell growth of both OE33 and OE19 (Fig. 2b). WST-1 assay showed that IGF-IR/dn blocked the growth of OE33 on plastic in a dose-dependent manner (Fig. 2c). IGF-IR/dn also reduced the number of colonies in a dose-dependent manner and strengthened the suppressive effect of cisplatin on colony formation of OE33 (Fig. 2d). Moreover, silencing IGF-IR by ad-shIGF-IR reduced colony number in a dose-dependent manner and enhanced cisplatin-induced suppression of colony formation in OE33 tumor cells.

Signaling analysis by Western blotting showed that ad-IGF-IR/dn could block both IGF-I- and IGF-II-induced phosphorylation of Akt in OE33 (Fig. 2e). IGF-IR/dn also reduced phosphorylation of both ERKs and S6. IGF-IR/dn could block des(1–3)IGF-I induced downstream signal transduction but not insulin-derived signals.

DNA fragmentation assays showed that IGF-IR/dn induced apoptosis in OE33 (Fig. 2f). In addition, IGF-IR/dn could enhance UV-induced apoptosis in OE33. The results were confirmed in another EAC cell, OE19. Moreover, ad-shIGF-IR showed almost the same effect as ad-IGF-IR/dn in both cell lines. Caspase-3 assays revealed that IGF-IR/dn up-regulated cisplatin-induced apoptosis in both OE33 and OE19 (Fig. 2g).

The results indicate that blockade of IGF-IR suppressed growth and colony formation and induced apoptosis in EAC cells.

The effect of IGF-IR/dn on ESCC cell growth

In the previous report, we showed the effects of IGF-IR/dn mainly for the ESCC cell line, TE1, so here we assessed the effect of IGF-IR blockade on several other ESCC cell lines as well [21].

IGF-BP3 suppressed proliferation of TE1 cultured in conditioned media with serum (Fig. 3a). The cell growth was markedly suppressed in the media without serum and IGF-I partially overcame this suppression. IGF-IR/482st suppressed in vitro growth of other ESCC cell lines, TE8, T.T, and T.Tn, in addition to TE1 (Fig. 3b). In every cell line, IGF-IR/dn was the most effective for growth suppression among tested inhibitors, wortmannin, LY294002, PD98059, and SB203580.

Soft agar assays revealed that IGF-IR/482st inhibited in vitro tumorigenicity in three ESCC cells: TE8, T.T, and T.Tn (Fig. 3c). In addition to IGF-IR/482st, another dominant negative, IGF-IR/950st, suppressed the carcinogenicity of T.Tn. Colony formation assays showed that IGF-IR/482st suppressed colony formation in a dose-dependent manner (Fig. 3d).

IGF-IR/dn blocked signal transduction in ESCC cell lines

Both IGF-I and IGF-II could induce phosphorylation of Akt-1 in both TE1 and TE8 cells (Fig. 4a). Effective concentrations of IGF-I were from 5 to 100 ng/ml, and IGF-II was also effective from 5 to 100 ng/ml. In both cell lines, 5 ng/ml IGF-I and 10 ng/ml IGF-II resulted in the activation of Akt-1 in 2.5 to 20 min (Fig. 4b).

Both Akt-1 and ERKs were phosphorylated by the ligands, IGF-I and IGF-II, in TE8 infected with control virus; however, Akt activation was blocked in the cells infected with IGF-IR/482st (Fig. 4c). The same results were observed in the other cell lines, TE1, T.T, and T.Tn (Fig. 4d–f). In the latter two cell lines, IGF-IR/482st inhibited the ligand-induced phosphorylation of S6. In T.Tn, des(1–3)IGF-I phosphorylated both downstream of Akt-1 and ERKs (Fig. 4g). In addition to IGF-IR/482st, IGF-IR/950st blocked phosphorylation of Akt-1 but not ERK in T.Tn.

Up-regulation of apoptotic induction on ESCC cell lines by IGF-IR/dn

DNA fragmentation assays revealed that the expression of IGF-IR/dn induced up-regulation of UV-induced apoptosis in TE8 (Fig. 5a). Annexin-V assays showed that IGF-IR/dn up-regulated 10% ethanol-induced early apoptosis in three cell lines, TE8, T.T, and T.Tn (Fig. 5b). Moreover, IGF-IR/dn increased apoptosis induced by both chemotherapies (cisplatin and 5-FU) in both TE8 and T.Tn (Fig. 5c). TUNEL assays confirmed the result that IGF-IR/482st enhanced cisplatin-induced apoptosis in both TE8 and TE1 (Fig. 5d). Both IGF-IR/482st and IGF-IR/950st up-regulated 5-FU-induced apoptosis in TE8 as detected by caspase-3 assays (Fig. 5e).

Both PD98059 and SB203580 up-regulated 5-FU-induced apoptosis in TE8 but wortmannin could not, as detected by annexin-V assays (Fig. 5f). Three inhibitors, wortmannin, LY294002, and SB203580, enhanced 10% ethanol-induced early apoptosis in T.Tn, but PD98059 did not.

The effect of IGF-IR on the migration of ESCC cell lines

T.T cells exhibited high mobility when cultured on plastic in a conditioned medium, but migration was reduced when these cells were cultured without serum (Fig. 6a). IGF-I stimulated the mobility of T.T in a dose-dependent manner, and IGFBP-3 reduced the migration ability of T.T cultured in conditioned media with FCS. The results indicated that the IGF/IGF-IR axis might play a part in the mobility of ESCC.

Both IGF-IR/dns suppressed the migration of T.T significantly (Fig. 6b). Moreover, both forms of IGF-IR/dn reduced the mobility of the other two cell lines, TE8 and T.Tn.

The effect of BMS-536924 for both types of esophageal carcinoma

The IGF-IR/InsR inhibitor, BMS-536924, blocked IGF-I-induced IGF-IR auto-phosphorylation and its down-stream signals, pAkt and pERKs, in an ESCC cell, TE8 (Fig. 7a). The same results were detected in an EAC cell, OE33. Compared to IGF-IR/dn, BMS-536924 could also block the phosphorylation of ERKs clearly in both cell lines.

BMS-536924 inhibited insulin-induced InsR autophosphorylation and activation of not only Akt but also ERKs in both cell types (Fig. 7b), unlike IGF-IR/482st and IGF-IR/950st.

The kinase inhibitor suppressed colony formation of TE8 completely and blocked that of OE33 in a dose-dependent manner (Fig. 7c). Caspase-3 assay showed that BMS-536924 enhanced 5FU-induced apoptosis in a dose-dependent manner (Fig. 7d).

The results indicate that IGF-IR target therapy might be a candidate strategy for both types of esophageal carcinomas.

Discussion

We show here that EAC cell lines express both IGF-II and IGF-IR, but not IGF-I, similar to ESCC. We also showed that IGF-IR was expressed in metastatic deposits in addition to the primary ESCC tumors. EAC expressed IGF-IR but tended to do so less frequently than ESCC. These results are compatible with the recent report in which higher IGF-IR protein expressions were observed in ESCC cells compared with EAC cells

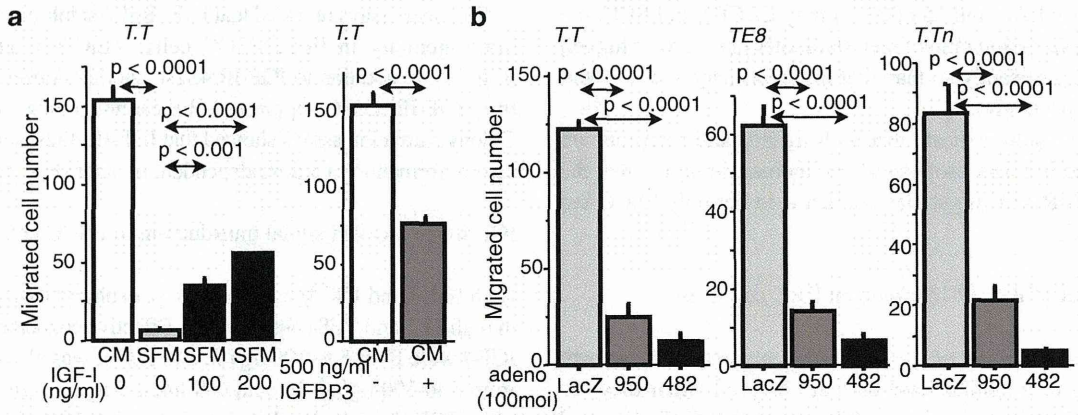


Fig. 6 The effect of IGF axis on migration of ESCC assessed by wounding assays. **a** TT cells were cultured with or without FBS ± IGF-I for 24 h and were cultured with/without IGFBP3. **b** Migration assay was performed for adenoviruses-infected cells

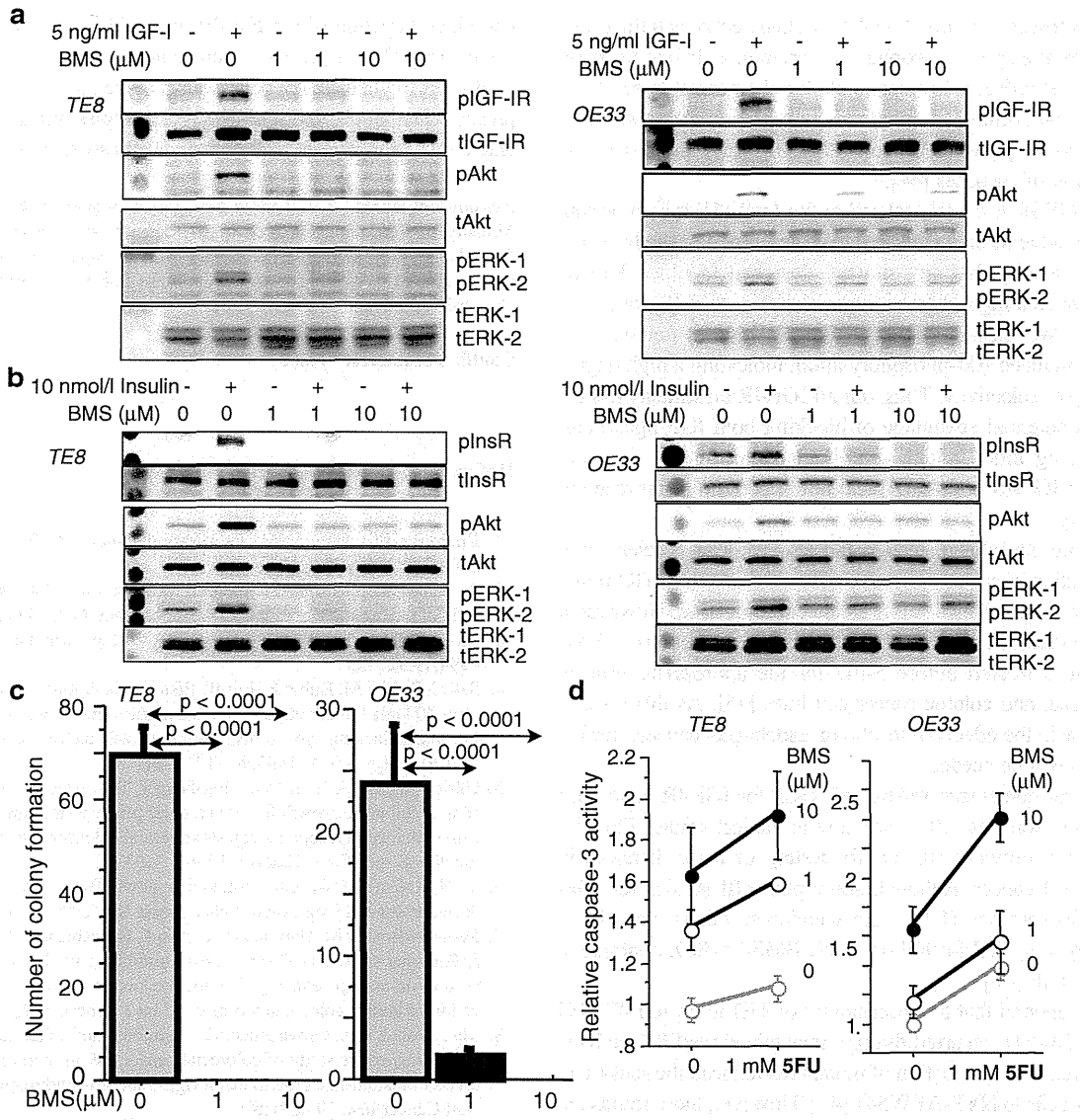


Fig. 7 The effect of BMS-536924 on both ESCC, TE8 and EAC, OE33. **a** After the cell was cultured with several amounts of BMS-536924, cells were stimulated for 5 min with IGF-I. Then Western blotting was performed. **b** After BMS-536924 treatment, the cells were stimulated for 5 min with insulin. **c** Colony formation assay revealed that this inhibitor reduced the number of colonies. **d** Caspase-3 assay revealed 5-FU-induced apoptosis

[22]. IGF-IR expression could also be useful as a novel prognostic marker for EAC [42]. Thus, IGF-IR might be a therapeutic target for many esophageal carcinomas.

In our previous study, we demonstrated that the IGF-IR axis is not only frequently overexpressed in ESCC and is associated with poor outcome but that it is also an exciting potential target for therapeutic intervention in this specific disease [21]. One of the possible mechanisms of IGF-IR overexpression in ESCC is that the miR-375 is downregulated by promoter methylation as miR-375 has a strong tumor-suppressive effect through inhibiting the expression of IGF-IR [43].

In this study, ad-IGF-IR/dn suppressed in vitro tumorigenicity, survival, and migration of both ESCC and EAC cells and also enhanced chemotherapy-induced apoptosis. In several cell lines representative of the two esophageal cancer subtypes (that express different patterns of IGF-IR and IGF ligand expression), the effects of ad-IGF-IR/dns were very similar, suggesting that IGF-IR targeting might have therapeutic potency for a variety of patients with esophageal carcinomas. This is also supported by the results from the multiple different inhibitors used in this study: IGF-IR/dns, shIGF-IR, and BMS-536924 all showed tumor-suppressive effects for esophageal carcinomas.

We showed here that IGF-IR blockade enhanced the effect of chemotherapy for esophageal carcinoma. It has been reported that IGF axis is responsible for chemoresistance. IGF-I inhibits 5-FU-induced apoptosis through increasing survivin levels, which prevents Smac/DIABLO release and blocks the activation of caspases [44].

As IGF-IR is closely related to the InsR [5], it is important to avoid adverse effects related to co-inhibition of the InsR and perhaps ideally that any strategy designed to block IGF-IR would have a high degree of specificity for IGF-IR compared to InsR. We show here that ad-IGF-IR/dn does not suppress insulin-induced Akt-phosphorylation, indicating a high degree of receptor selectivity. Thus, our ad-IGF-IR/dn strategy has the distinct potential advantage of blocking both IGF ligand signals, being independent of IGFBPs, interrupting signaling between IGF-IR and Akt-1, and not affecting insulin receptor signaling.

On the other hand, InsR could also work as accelerator of proliferation in cancer cells. Thus, the dual targeting TKI might have some advantages to block cancer progression. However, it was reported that insulin enhances anticancer functions of 5-FU when it is treated before 5-FU for the appropriate time in esophageal and colonic cancer cell lines [45]. As there is discrepancy in the effects of insulin on esophageal cancers, further analysis will be needed.

Several humanized mAbs and TKIs for IGF-IR have been generated, some of which are now in clinical studies [26–28]. This study provides support for testing of these therapies in esophageal cancer. Although some phase III studies for IGF-IR mAbs (but not TKIs) were withdrawn, others including a dual targeting TKI for IGF-IR/InsR, BMS-754807, continue in clinical trials [46].

It is reported that the insensitivity of TE1 to an IGF-IR TKI NVP-AEW541 occurred through maintained ras/ERK activity. Moreover, the transduction of mutant ras reduced the sensitivity of TE-1 cells to NVP-AEW541 [47]. However, these results are different from our reported data that NVP-AEW541 inhibited the cancer progression of four gastrointestinal cancer cell lines, including TE-1 [48]. It would be interesting to analyze the reasons for the differences between these studies.

In addition, we have reported an IGF-IR mAb, figitumumab (CP-751,871), that could suppress gastrointestinal cancers expressing k-ras mutations, including TE-1 [49]. Further studies are needed to assess the effect and mechanism of IGF-IR blockade in k-ras mutated cancers.

In this study, we showed that a dual IGF-IR/InsR TKI is effective for both types of human esophageal carcinomas. Several advantages of dual targeting strategies for esophageal carcinoma have been reported. TAE226, a dual tyrosine kinase inhibitor for FAK and IGF-IR, could suppress Barrett's EAC [50]. The combination of Her2 mAb, trastuzumab, and IGF-IR mAb, α-IR3, was more effective in inhibiting in vitro proliferation of EAC than treatment with either agent alone [42]. Thus, combined targeting of the IGF-IR axis with these other tumor drivers may show significant therapeutic promise.

IGF-IR might therefore be important in the progression of esophageal carcinomas, and IGF-IR target therapies might be candidate options for patients with both types of esophageal cancers.

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Conflicts of interest None

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (8): Gastric cancer

An updated review of gastric cancer in the next-generation sequencing era: Insights from bench to bedside and *vice versa*

Hiroyuki Yamamoto, Yoshiyuki Watanabe, Tadateru Maehata, Ryo Morita, Yoshihito Yoshida, Ritsuko Oikawa, Shinya Ishigooka, Shun-ichiro Ozawa, Yasumasa Matsuo, Kosuke Hosoya, Masaki Yamashita, Hiroaki Taniguchi, Katsuhiko Nosho, Hiromu Suzuki, Hiroshi Yasuda, Yasuhisa Shinomura, Fumio Itoh

Hiroyuki Yamamoto, Yoshiyuki Watanabe, Tadateru Maehata, Ryo Morita, Yoshihito Yoshida, Ritsuko Oikawa, Shinya Ishigooka, Shun-ichiro Ozawa, Yasumasa Matsuo, Kosuke Hosoya, Masaki Yamashita, Hiroshi Yasuda, Fumio Itoh, Division of Gastroenterology and Hepatology, Department of Internal Medicine, St. Marianna University School of Medicine, Kawasaki 216-8511, Japan
Hiroaki Taniguchi, Division of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan
Katsuhiko Nosho, Yasuhisa Shinomura, Department of Gastroenterology, Rheumatology and Clinical Immunology, Sapporo Medical University School of Medicine, Sapporo 060-8543, Japan
Hiromu Suzuki, Department of Molecular Biology, Sapporo Medical University School of Medicine, Sapporo 060-8556, Japan
Author contributions: Yamamoto H conceived the topic, reviewed the literature, and prepared the manuscript; Watanabe Y, Maehata T, Morita R, Yoshida Y, Oikawa R, Ishigooka S, Ozawa S, Matsuo Y, Hosoya K, Yamashita M, Taniguchi H, Nosho K, and Suzuki H reviewed and analyzed the literature; Yasuda H, Shinomura Y and Itoh F provided intellectual support.
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Correspondence to: Hiroyuki Yamamoto, MD, FJSIM, PhD, Division of Gastroenterology and Hepatology, Department of Internal Medicine, St. Marianna University School of Medicine, Sugo 2-16-1 Miyamae, Kawasaki 216-8511, Japan. h-yama@marianna-u.ac.jp
Telephone: +81-44-9778111 Fax: +81-44-9762282
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Abstract

Gastric cancer (GC) is one of the most common malignancies and remains the second leading cause of cancer-related death worldwide. There is an increasing understanding of the roles that genetic and epigenetic alterations play in GCs. Recent studies using next-generation sequencing (NGS) have revealed a number of potential cancer-driving genes in GC. Whole-exome sequencing of GC has identified recurrent somatic mutations in the chromatin remodeling gene *ARID1A* and alterations in the cell adhesion gene FAT4, a member of the cadherin gene family. Mutations in chromatin remodeling genes (*ARID1A*, *MLL3* and *MLL*) have been found in 47% of GCs. Whole-genome sequencing and whole-transcriptome sequencing analyses have also discovered novel alterations in GC. Recent studies of cancer epigenetics have revealed widespread alterations in genes involved in the epigenetic machinery, such as DNA methylation, histone modifications, nucleosome positioning, noncoding RNAs and microRNAs. Recent advances in molecular research on GC have resulted in the introduction of new diagnostic and therapeutic strategies into clinical settings. The anti-human epidermal growth receptor 2 (HER2) antibody trastuzumab has led to an era of personalized therapy in GC. In addition, ramucirumab, a monoclonal antibody targeting vascular endothelial growth factor receptor (VEGFR)-2, is the first biological treatment that showed survival benefits as a single-agent therapy in patients with advanced GC who progressed after first-line chemotherapy. Using NGS to systematically identify gene alterations in GC is a promising approach with remarkable potential for investigating the pathogenesis of GC and identifying novel therapeutic targets, as well as useful biomarkers. In this review, we will summarize the recent advances in the understanding of the molecular pathogenesis of GC, focusing on the potential use of these genetic and epigenetic alterations as diagnostic biomarkers and novel therapeutic targets.

Key words: Next-generation sequencing; Microsatellite instability; MicroRNA; Epigenetic field defect; Gastric washes; Insulin-like growth factor 1 receptor

Core tip: The genetic and epigenetic alterations in gastric cancers (GC) have biological and clinical implications. Recent advances in the molecular research of GC have introduced new diagnostic and therapeutic strategies to clinical settings. In this review, we summarize the key findings of past reports pertaining to the genetics and epigenetics of GC and their relationship to and future applications in next-generation sequencing (NGS). We also describe the recurrently mutated genes and alterations in GC identified by NGS technology and discuss the basic framework for future investigations, including the challenges of using NGS as a tool for biomarker and therapeutic target discovery.

Yamamoto H, Watanabe Y, Maehata T, Morita R, Yoshida Y, Oikawa R, Ishigooka S, Ozawa S, Matsuo Y, Hosoya K, Yamashita M, Taniguchi H, Nosho K, Suzuki H, Yasuda H, Shinomura Y, Itoh F. An updated review of gastric cancer in the next-generation sequencing era: Insights from bench to bedside and *vice versa*. *World J Gastroenterol* 2014; 20(14): 3927-3937 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i14/3927.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i14.3927

INTRODUCTION

Gastric cancer (GC) is the second highest cause of global cancer mortality. GC is a heterogeneous disease with multiple environmental etiologies and alternative pathways of carcinogenesis[1,2]. One of the major etiologic risk factors for GC is *Helicobacter pylori* (*H. pylori*) infection, but only a small proportion of individuals infected with *H. pylori* develop GC[3,4]. There is an increasing understanding of the roles that genetic and epigenetic alterations play in GCs (Figure 1). Consequently, the development of appropriate biomarkers that reflect an individual's cancer risk is essential to reduce the mortality from GC[5,6]. Recent advances in molecular research of GC have brought new diagnostic and therapeutic strategies into clinical settings.

Next-generation sequencing (NGS) is a technology that involves the parallel sequencing of enormous amounts of short DNA strands from randomly fragmented copies of a genome[7,8]. NGS methods used for genome[9], exome[10], epigenome[11] and transcriptome[12] sequencing have the potential to provide novel avenues towards achieving a comprehensive understanding of diseases, including cancer[13,14]. Such advances have also shown puzzling tumor heterogeneity with limited somatic alterations shared between tumors of the same histopathologic subtype[15-17]. Although NGS techniques are just beginning to expand our abilities to detect genome-wide alterations in GC, several NGS studies in GC have recently been published[18].

In this review, we summarize the key findings of past reports pertaining to the genetics and epigenetics of GC and their relationship to and future application in NGS. We also describe the recurrently mutated genes and alterations in GC identified by NGS technology and discuss the basic framework for future investigations, including the challenges of using NGS as a tool for biomarker and therapeutic target discovery.

MICROSATELLITE INSTABILITY

A type of genetic instability characterized by alterations in length within simple repeat microsatellite sequences, termed microsatellite instability (MSI), occurs in approximately 15% of sporadic GCs, mainly as a result of epigenetic changes[19-22]. Genetic and epigenetic inactivation of DNA mismatch repair (MMR) genes leads to the mutator phenotype, mutations in cancer-related genes and cancer development (Figure 2). MSI underlies a distinctive carcinogenic pathway because MSI-positive (MSI^+) GCs exhibit many differences in clinical, pathological and molecular characteristics compared with MSI-negative (MSI^-) GCs[19-22]. The differences in genotype occur because defective MMR results in a strong mutator phenotype with a very specific mutation spectrum. MSI mainly accumulates frameshift mutations in the repeated sequences located in the coding regions of a target tumor suppressor or other tumor-related genes[23-26]. The atypical genotype of MSI^+ GCs also includes specific patterns of gene dysregulation. MSI^+ GCs often show epigenetic alterations, such as hypermethylation of various genes, including the key MMR gene *MLH1*. The differences in genotype and phenotype between MSI^+ and MSI^- GCs are likely linked to their differences in biological and clinical features. Recent findings from NGS analysis, such as the frequent mutation of the AT-rich interactive domain 1A (ARID1A) in MSI^+ GCs, support this notion[27,28].

The clinicopathological, genetic, epigenetic, prognostic and therapeutic characteristics of MSI^+ GCs are becoming clearer, but further research is still required. Because molecular targeting therapeutics are being used in clinical settings and trials, the differential regulation of molecular target genes in MSI^+ and MSI^- GCs[29,30] needs to be clarified. Diagnostic characterization of the MSI status of GCs thus has important implications for basic and clinical oncology.

Frequent inactivating mutations of ARID1A in molecular subtypes of GC identified by exome sequencing

Holbrook *et al*[31] analyzed 50 GC samples with targeted deep sequencing of the DNA of 384 genes. In addition to the previously reported mutations in genes belonging to various pathways, the authors found tractable target genes, such as the genes for the thyrotropin receptor and the Rho-associated coiled-coil containing protein kinases ROCK1 and ROCK2. Wang *et al*[27] performed exome

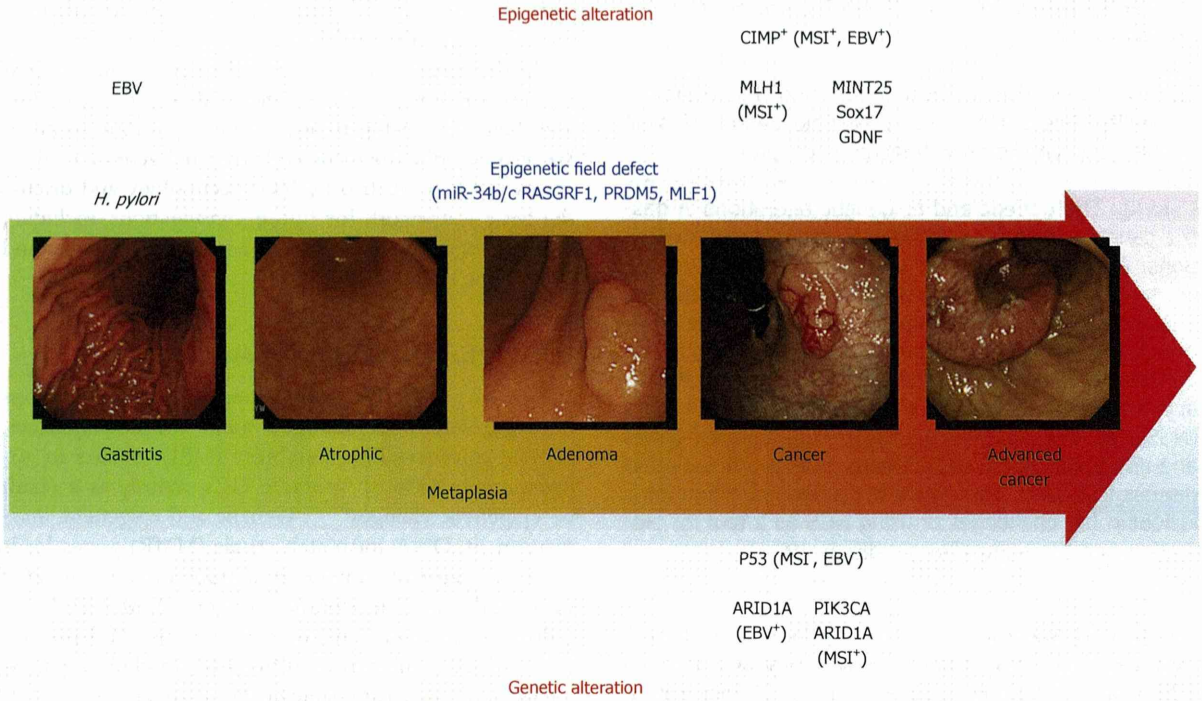

Figure 1 Genetic and epigenetic alterations in gastric carcinogenesis. The model for gastric carcinogenesis is presented based on genetic and epigenetic alterations. Methylation of the genes in blue appears to be involved in an epigenetic field defect. *H. pylori*: *Helicobacter pylori*; MSI: Microsatellite instability; EBV: Epstein-Barr virus; CIMP: CpG island methylator phenotype.

sequencing of 22 GC samples and found novel mutated genes and pathway alterations involved in chromatin modification. A validation study confirmed frequent inactivating mutations or protein loss of the ARID1A gene, which encodes one of the subunits in the Switch/Sucrose Nonfermentable (SWI-SNF) chromatin remodeling complex. The mutation spectrum for ARID1A differed among molecular subtypes of GC; mutations were detected in 83% of GCs with MSI, 73% of GCs with EBV infection and 11% of GCs without EBV and MSI. Moreover, ARID1A mutations were negatively associated with TP53 mutations. ARID1A alterations were associated with better prognosis in a stage-independent manner. These results suggest the importance of altered chromatin remodeling in the pathogenesis of GC.

Recurrent somatic mutations in cell adhesion and chromatin remodeling genes identified by exome sequencing

Zang *et al*[28] also analyzed a spectrum of somatic alterations in GC by sequencing the exomes of 15 GC specimens, including 11 intestinal-type, 1-mixed-type, and 3 diffuse-type adenocarcinomas and their matched normal DNAs. TP53 (11/15 tumors), PIK3CA (3/15) and ARID1A (3/15) were frequently mutated. Among the frequently mutated genes, cell adhesion was the most significant biological pathway affected. A prevalence screening confirmed mutations in FAT4, a member of the cadherin gene family, in 5% of GCs (6/110) and FAT4 genomic deletions in 4% (3/83) of GCs. Mutations in chromatin remodeling genes (*ARID1A*, *MLL3* and *MLL*) were also found in 47% of GCs. ARID1A mutations were detected in 8% of GCs (9/110) and were associated with concurrent PIK3CA mutations and MSI. Both FAT4 and ARID1A showed tumor-suppressor activity in functional assays. Somatic inactivation of FAT4 and ARID1A may thus be key tumorigenic events in a subset of GCs. Because PI3K inhibitors are currently in clinical testing as treatment for GC[32], it will be interesting to evaluate whether the tumor responses to these compounds are affected by the genomic status of ARID1A.

Frequent loss of ARID1A expression in GC with EBV infection or MSI

Mutations of ARID1A lead to a loss of protein expression in GC and are particularly associated with EBV infection or MSI. Abe *et al*[33] investigated the significance of the loss of ARID1A in 857 GC cases, including 67 EBV^+ and 136 MLH1-lost MSI^+ GCs. Loss of ARID1A expression was significantly more frequent in cases of EBV^+ (23/67; 34%) and MSI^+ (40/136; 29%) GCs than in cases of EBV/MSI^- (32/657; 5%) GCs. Loss of ARID1A was correlated with larger tumor size, deeper depth of invasion, lymph node metastasis and poorer prognosis in cases of EBV/MSI^- GC. A correlation with tumor size and diffuse-type histology was found only in the MSI^+ GC; no correlation was observed in EBV^+ GC. Loss of ARID1A expression in EBV^+ GC was frequent in the early stage of GC, but EBV infection did not cause loss of ARID1A in GC cell lines. Thus, loss of ARID1A may be an early event in EBV^+ GC and may precede EBV infection in gastric epithelial cells. On the other hand,

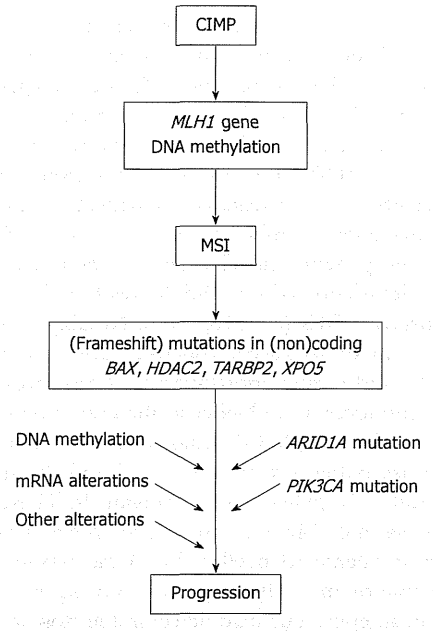

Figure 2 Molecular pathway for microsatellite instability+ gastric cancer. The model for the carcinogenesis of microsatellite instability (MSI)$^+$ gastric cancer is presented. CIMP: CpG island methylator phenotype.

loss of ARID1A may be involved in the progression of EBV/MSI$^-$ GCs. Thus, loss of ARID1A appears to have different, pathway-dependent roles in GC.

WHOLE-GENOME SEQUENCING ANALYSIS OF GC

To explore the complete list of somatic alterations in GC, Nagarajan *et al*[34] combined massively parallel short read and DNA paired-end tag sequencing for the first whole-genome analysis of two GCs, one with CIN and the other with MSI. Integrative analysis and de novo assemblies revealed the architecture of a wild-type KRAS amplification, a common driver event in GC[35]. Three distinct mutational signatures were discovered against a genome-wide backdrop of oxidative and MSI-associated mutational signatures. Combining sequencing data from 40 complete GC exomes and targeted screening of an additional 94 independent GCs led to the discovery of ACVR2A, RPL22 and LMAN1 as recurrently mutated genes in MSI$^+$ GC and the identification of PAPPA as a recurrently mutated gene in TP53 wild-type GC. These results highlight how whole-genome sequencing analysis can provide relevant information about tissue-specific carcinogenesis that would otherwise be missed in exome-sequencing data. WGS of more GCs will uncover more recurrently altered genes.

miRNA alterations

A microRNA (miRNA) is a small noncoding RNA that regulates gene expression at the posttranscriptional level and is critical in many biological processes and cellular pathways[36-40]. The causes of aberrant miRNA expression patterns in cancer include DNA copy number amplification or deletion, inappropriate transactivation, transcriptional repression by oncogenic and other factors, failure of miRNA post-transcriptional regulation and genetic mutation or transcriptional silencing associated with hypermethylation of the CpG island promoters.

There is accumulating evidence to support the notion that miRNA alterations play a key role in the pathogenesis of GC[41-44]. A large number of miRNAs with different biological functions have been found to be altered and correlated with clinicopathological characteristics and/or prognosis in GC. Moreover, the clinical potential of miRNA alterations as minimally invasive diagnostic biomarkers and therapeutic targets has been extensively reported[37,40,42,44]. Recent studies have shown that tumor-derived miRNAs are present and stable in circulation, and the levels of circulating miRNAs are detectable and quantifiable. Both tissue and soluble miRNAs are candidates for diagnostic biomarkers and therapeutic targets in GCs[44]. The basic strategy of current miRNA-based treatment studies is to either antagonize the expression of target oncogenic miRNAs with antisense therapy and other technology or to restore the function of impaired tumor suppressor miRNAs[42].

The inclusion of different isoforms of miRNA (isomiRs) that are natural variants of mature miRNAs will form a detailed miRnome. Because expression of isomiRs can be estimated by NGS, NGS platforms provide the most effective method of miRNA profiling, leading to the identification of the miRNA alterations with clinical applications. Li *et al*[45] sequenced small RNAs from one pair of GC and noncancerous tissue and found that isomiR patterns are significantly different between these tissues. Moreover, these authors found that the 5p arm and 3p arm miRNAs derived from the same pre-miRNAs have different tissue preferences in GC and noncancerous tissue, suggesting a novel mechanism regulating mature miRNA selection.

WHOLE-TRANSCRIPTOME SEQUENCING OF GC

The first comprehensive RNA-seq study in GC has been recently published. Kim *et al*[46] applied a whole-transcriptome sequencing approach to 24 GC samples and six noncancerous tissue specimens. Importantly, these authors developed a multilayered integrative analysis to identify various types of transcriptional aberrations, such as differentially expressed mRNAs and miRNAs, as well as recurrently mutated genes. A central metabolic regulator gene, AMPKa2 (PRKAA2), was identified as a potential functional target in GC. Six key miRNAs (miR-548d-3p, miR-20b, miR-135b, miR-140-3p, miR-93 and miR-19a) in GC were also identified.

Epigenetic alterations

Epigenetic regulation is essential for the normal develop-

ment and maintenance of tissue-specific gene expression patterns in mammals. Disruption of epigenetic regulation can lead to altered gene function and malignant cellular transformation[47]. Recent cancer epigenetic studies have revealed various alterations in the epigenetic machinery in GC, including DNA methylation, histone modifications, nucleosome positioning, noncoding RNAs and miRNAs[48-52]. Aberrant DNA methylation in the promoter CpG islands of genes results in inactivation of tumor suppressor and other tumor-related genes in cancer cells and is the most well-defined epigenetic hallmark in GC. Methylation of a large number of genes with different biological functions has been found to be correlated with the clinicopathological characteristics and prognosis in GC[48-52]. DNA methylation with its advantages as a biomarker for the detection of cancer in biopsy specimens and body fluids that can be obtained non-invasively, such as serum and gastric washes, may have a clinical application in GC. Detection of aberrant DNA methylation of genes, such as *CDH1*, *DAPK*, *GSTP1*, *p15*, *p16*, *RARβ*, *RASSF1A*, *RUNX3* and *TFPI2*, in the serum may be a useful biomarker for the detection of GC[50]. Studies of DNA methylation and histone modification using NGS technologies, such as whole-genome bisulfite sequencing and targeted bisulfite sequencing, will lead to new discoveries and improve our knowledge of the epigenomics of GC[11].

Association of the aberrant methylation of RASGRF1 with an epigenetic field defect and an increased risk of GC

Aberrant DNA methylation is implicated in the epigenetic field defect seen in GC. Thus, it is important to identify predictive biomarkers by screening for DNA methylation in the noncancerous background gastric mucosa of patients with GC. Using methylated-CpG island amplification coupled with CpG island microarray (MCAM) analysis, Takamaru *et al*[53] found 224 genes that were methylated in the noncancerous gastric mucosa of patients with GC. Among them, RASGRF1 methylation was significantly elevated in the gastric mucosa from patients with either intestinal- or diffuse-type GC, compared with the mucosa from healthy individuals. RASGRF1 methylation was independent of mucosal atrophy and could be used to distinguish both serum pepsinogen test-positive and -negative patients with GC from healthy individuals. Ectopic expression of RASGRF1 suppressed colony formation and Matrigel invasion by GC cells. RASGRF1 methylation appears to be significantly involved in the epigenetic field defect of the stomach and to be a useful biomarker to identify individuals at high risk for GC.

Association of aberrant methylation of miR-34b/c with an epigenetic field defect and an increased risk of GC

The silencing of miRNAs is often associated with CpG island hypermethylation. Thus, to identify epigenetically silenced miRNAs in GC, Suzuki *et al*[54] screened for miRNAs that were induced by treatment of GC cells with 5-aza-2'-deoxycytidine and 4-phenylbutyrate. Hypermethylation of the neighboring CpG island epigenetically silenced miR-34b and miR-34c. Methylation of the miR-34b/c CpG island was frequently observed in GC cell lines (13/13, 100%) but not in normal gastric mucosa from healthy *H. pylori*-negative individuals. Transfection of the precursors of miR-34b and miR-34c into GC cells suppressed growth and changed the gene expression profile. Methylation of miR-34b/c was found in a majority of primary GCs (83/118, 70%). Notably, analysis of the non-cancerous gastric mucosae from GC patients (n = 109) and healthy individuals (n = 85) revealed that methylation levels were higher in the gastric mucosae of patients with multiple GC lesions than in the mucosae from those patients with single GC and the mucosae from healthy *H. pylori*-positive individuals. These results suggest that miR-34b and miR-34c are novel tumor suppressors frequently silenced by DNA methylation in GC. Methylation of miR-34b/c appears to be significantly involved in an epigenetic field defect in the stomach and to be a useful biomarker to identify individuals at high risk for multiple GC.

Methylation of miR-34b/c in the mucosa of the noncancerous gastric body may be a useful biomarker for predicting the risk of metachronous GC

Metachronous GC can develop after endoscopic resection of GC and is not predictable based on the clinical characteristics alone. Aberrant DNA methylation in noncancerous gastric mucosa has been implicated in gastric carcinogenesis and may be a useful biomarker of GC risk. Suzuki *et al*[55] evaluated the clinical utility of DNA methylation as a biomarker of metachronous GC risk. Scheduled follow-up endoscopy was performed in 129 patients after curative endoscopic resection of early GC. Biopsy specimens were collected from noncancerous mucosa in the gastric antrum and body. A quantitative methylation analysis of miR-34b/c, SFRP1, SFRP2, SFRP5, DKK2 and DKK3 using bisulfite pyrosequencing was performed on the collected biopsy specimens. The utility of the methylation status for predicting the risk of developing metachronous GC was analyzed using Kaplan-Meier and Cox proportional hazards models. During the follow-up period, 17 patients (13%) developed metachronous GCs. The cumulative incidence of metachronous GC was significantly higher among patients with elevated miR-34b/c, SFRP2 and DKK2 methylation in the gastric body. Elevated methylation of miR-34b/c showed the most significant association with the risk of metachronous GC; the cumulative incidence of metachronous GC was much higher in the high miR-34b/c-methylation group than in the low methylation group. Multivariate analysis adjusted for age, sex, *H. pylori* status and pathological findings showed that miR-34b/c methylation in the gastric body was an independent predictor of metachronous GC risk. Methylation of miR-34b/c in the mucosa of the noncancerous gastric

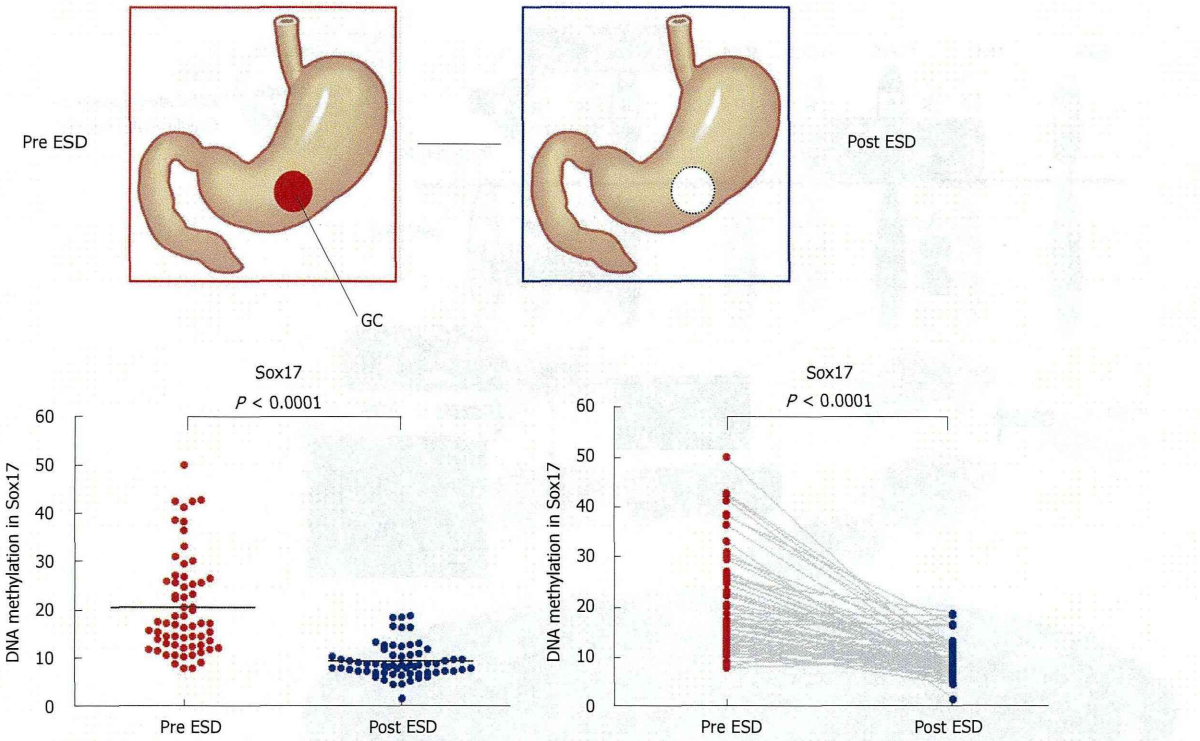

Figure 3 Methylation levels of Sox17 before and after endoscopic submucosal dissection. Methylation levels of Sox17 were analyzed by pyrosequencing using the DNA recovered from gastric washes before and after endoscopic submucosal dissection[57].

body may be a useful biomarker for predicting the risk of metachronous GC. Finally, NGS technologies may characterize an epigenetic field defect more clearly and highlight more useful biomarkers.

Sensitive and specific detection of early GC by DNA methylation analysis of gastric washes

Because many mucosal cells can be found in the gastric juice, the detection of molecular markers in the gastric juice was a possible noninvasive approach to detect GC. However, the use of gastric juice as a molecular diagnostic or predictive tool has been previously reported to be impractical because the DNA is easily degraded by gastric acidity. In this regard, Watanabe *et al*[56] have developed a new method for GC detection by DNA methylation in gastric washes but not in gastric juice. These authors analyzed 51 candidate genes in 7 GC cell lines and 24 GC samples (training set). They then selected 6 genes (*MINT25*, *RORA*, *GDNF*, *ADAM23*, *PRDM5* and *MLF1*) for further analyses. The methylation status of these genes was analyzed in a test set consisting of 131 GCs at various stages. The 6 candidate genes were validated in a different population of 40 primary GC samples and 113 noncancerous gastric mucosa samples. The 6 genes showed differential methylation in GC and normal mucosa in the training, test and validation sets. GDNF and MINT25 were the most sensitive molecular markers of early-stage GC, whereas PRDM5 and MLF1 were markers of a field defect. A close correlation between methylation levels in tumor biopsy samples and gastric washes was noted. MINT25 methylation showed the best sensitivity (90%) and specificity (96%), and it had the greatest area under the receiver operating characteristic curve (0.961) in terms of tumor detection in gastric washes. MINT25 methylation in gastric washes may be a sensitive and specific marker for the screening of GC.

Detection of early GC by DNA methylation analysis of Sox17 in gastric washes

Although minimally invasive treatment is widely accepted for early-stage GC, appropriate risk markers to detect residual cancer after endoscopic resection and the potential for recurrence are not available. To find candidate genes that might be markers for the detection of early GC, Oishi *et al*[57] performed methylated CpG island amplification microarray analysis on 12 gastric washes (from the pre- and post-endoscopic treatment of six patients). Among the candidate genes, the *Sox17* gene was selected for further analysis. The DNA methylation status of Sox17 was examined in a validation set consisting of 128 gastric wash samples (64 pre-treatment and 64 post-treatment) from cases of early GC. Sox17 showed significant differential methylation in the pre- and post-treatment gastric washes of early GC patients (Figure 3). Moreover, the treatment of GC cells that lacked Sox17 expression with the methyltransferase inhibitor 5-aza-2'-deoxycytidine restored the gene's expression. Additionally, the introduction of exogenous Sox17 into silenced