IND 申請者は 21 CFR 312.33 の定めるところにより年次報告書（Annual report）を作成して FDA に提出しなくてはならない。この年次報告書は記載すべき７つの要素が定められている。そのうち，安全性情報に関する事項は２項目が規定されている。一点は「臨床，非臨床試験両者の全ての試験における有害事象，死亡，安全性報告，脱落の総括的なサマリーを含む情報」であり，もう一点は「動物実験での動性試験あるいは，当該の被検薬もしくは類似薬の臨床試験結果から考えられる特別な重篤性の危険性」である。通常，上記の FDA への書面・迅速報告を要求されない有害事象は年次報告書の integrated summary にまとめられ，書面にての報告や迅速報告を行った有害事象は，簡略な要約とともに事象としての特異性，被検薬との関連性，注意事項等を考察して記載がなされる。通常，迅速報告に該当しない有害事象の報告はこの年次報告書もしくは IND 関連情報の改定時にまとめて報告される。

3. 市販後の副作用報告

市販後の副作用報告は 21 CFR 314.80 “Postmarketing reporting of adverse drug experiences” で規定されている。この規定では海外からの報告，すなわち日本国内で発生した副作用情報は文献上の報告と同じく，重篤（serious）かつ添付書に記載されていない（unlabeled）有害事象が報告の対象とされている。このため，FDA に報告すべきか否かの判断は，添付書の記載の有無，添付書に記載されている副作用と比較して，重症度，特異性において異なるかどうかで判断することとなる。この場合の添付書は米国における添付文書の副作用に関する記載内容であり，日本での添付文書ではない。また，米国内での市販後臨床試験における有害事象は因果関係を有するものだけでよいこととなっている。用語の定義に関しては 21 CFR 314.80 で規定されているが，これらの定義は IND を規定している 21 CFR 312.32 と同様である。市販後の報告義務は 21 CFR 314.50 で規定される承認申請者（applicant）が負うこととなっている。

重篤かつ添付書に記載されていない有害事象の報告を applicant が受けとった場合には，米国の内外を問わず（日本からの報告を含めて），情報を受けとった日より 15 暦日以内に FDA に報告書を “ postmarketing 15-day : Alert reports” として報告しなければならない。

Postmarketing における報告も，基本的には FDA Form 3500A を使用するが，書式の記載方法は IND の場合とほぼ同様である。FDA form 3500A の “ G.: All manufacturers, 5.” では “ Pre-1938” の項目があるが，これは 1938 年以降に NDA（New Drug Application）による承認制度以前より使用されていたいわゆる “grand father drug” と区別する目的で存在する。

4. FDA における英文

FDA はいわゆる政府機関ではあるが，報告書作成においては書式や形式に必要以上にとらわれることなく，実際的に運用する傾向にあり，また，記載方法に関して疑問や問題が生じた場合には IND の担当 CSO/PM や受付担当部署が質問等には迅速かつ適切に返答してくれる。また，クリントン大統領政権下で出された “ plain English” を FDA では実践しており，また，その遵守を reviewer も期待されており，スポンサー側も英文作成にあたっては必要以上に修辞的な文章を作成する必要はない。

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

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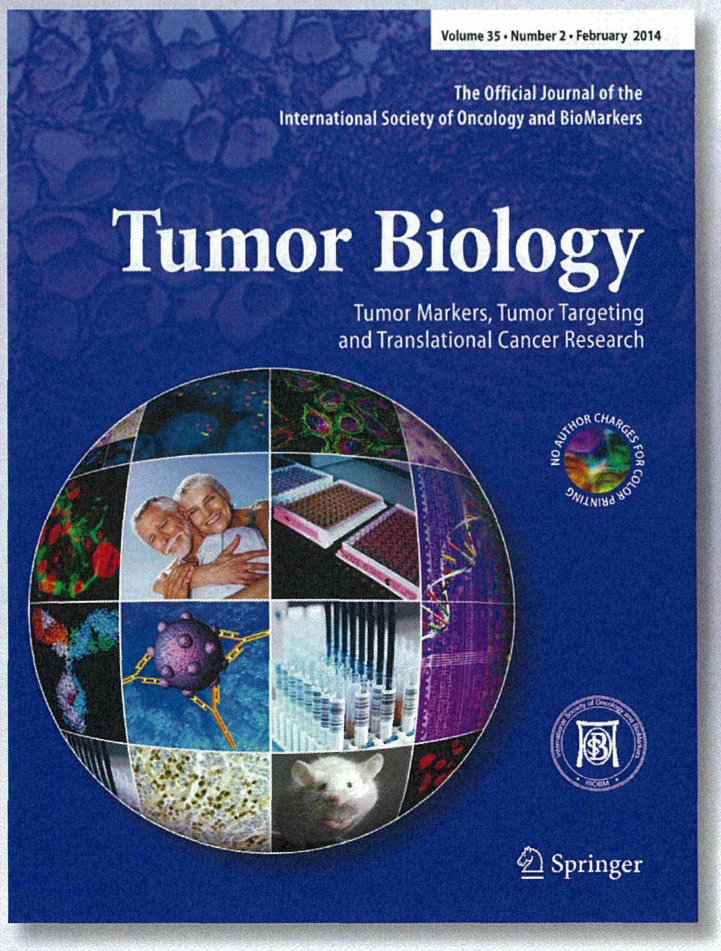

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RESEARCH ARTICLE

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.

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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,

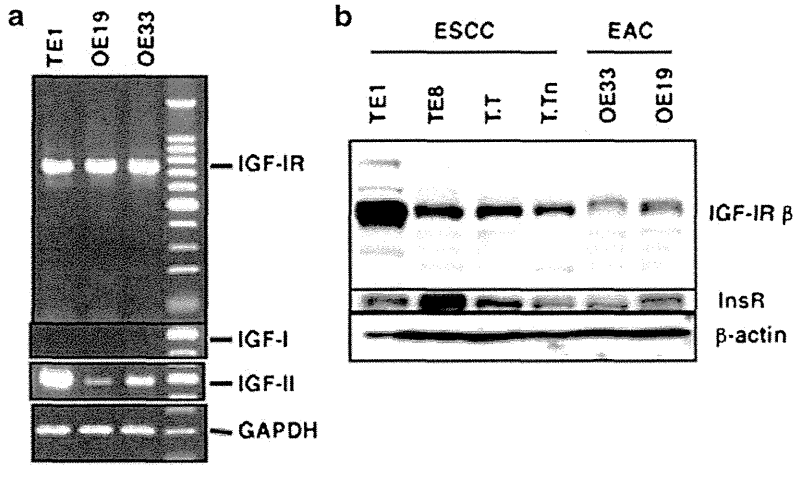

Fig. 1 The expressions of IGF-axis in esophageal carcinoma cell lines. **a** RT-PCR revealed that three cells express mRNAs of IGF-II and IGF-IR but not IGF-I. **b** Western blotting showed that two EAC and four ESCC cells express both IGF-IR and InsR

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%	

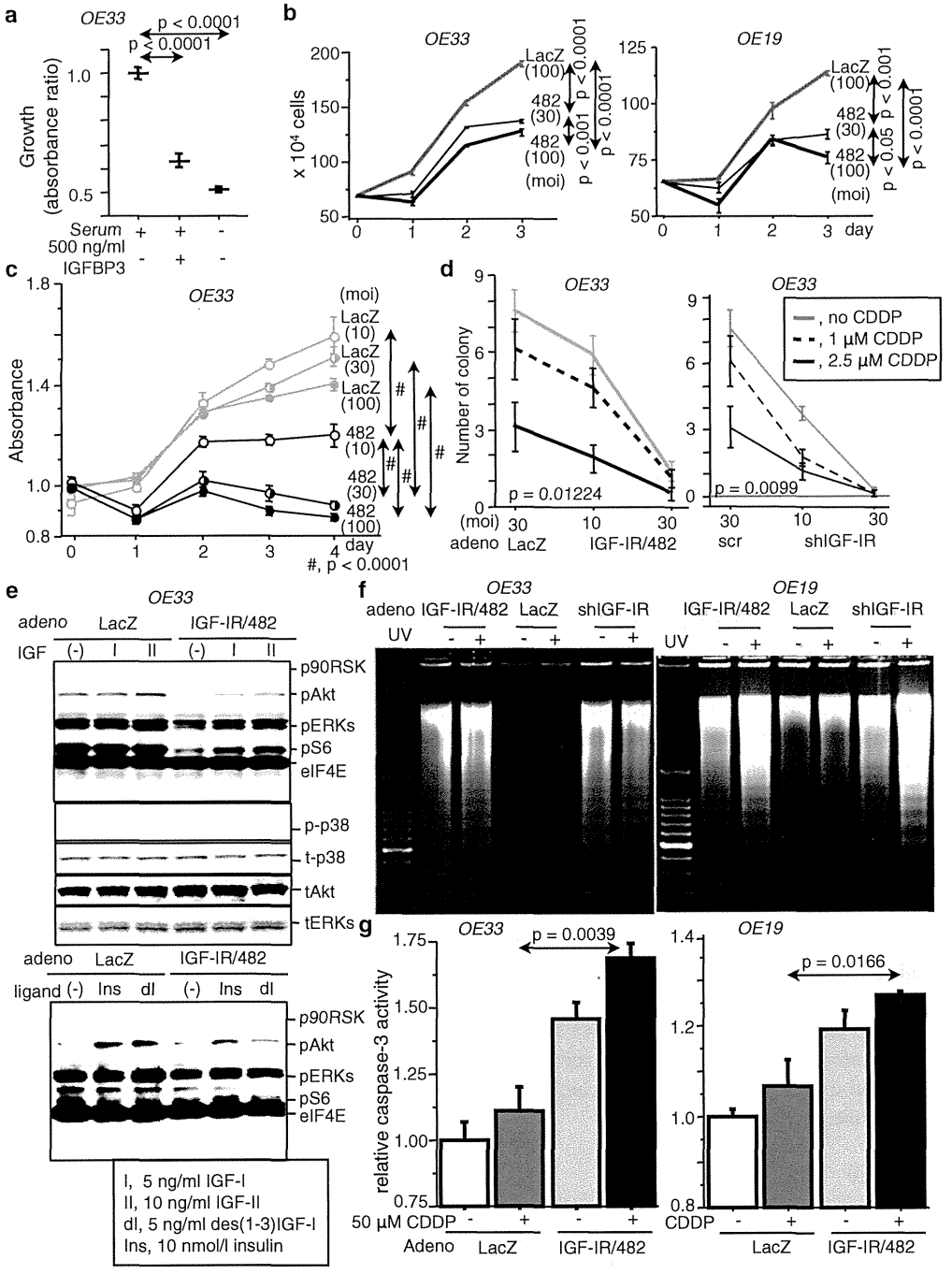

Fig. 2 The effect of IGF-IR on EAC cells. **a** WST-1 assay showed cell growth of OE33, 48 h of culture with/without IGFBP3. **b** Trypan blue assay showed the number of viable cells. **c** WST-1 assay revealed cell proliferation of adenoviruses-infected OE33. **d** Colony formation assays showed the 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].

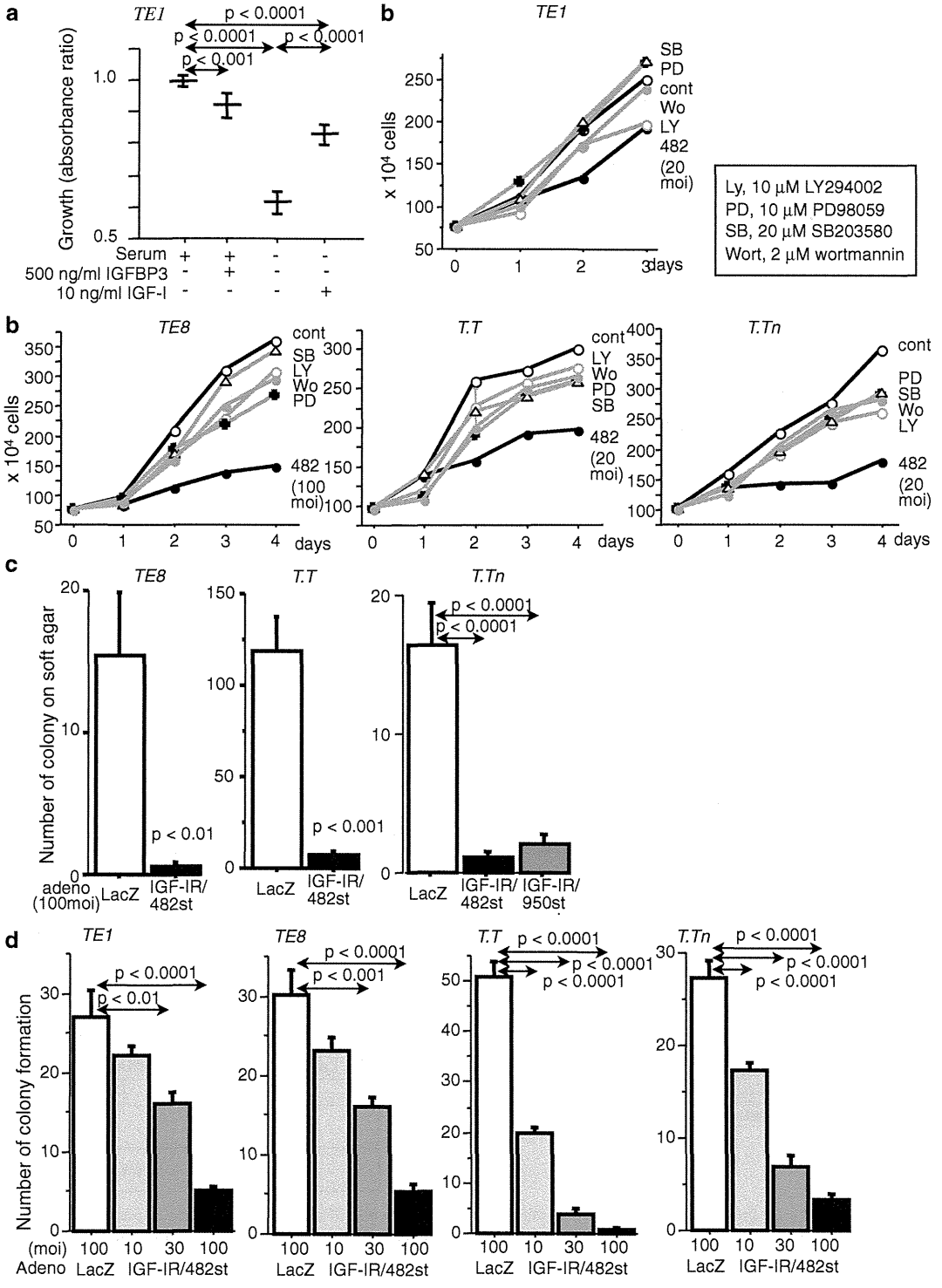

Fig. 3 The effect of IGF-IR on the growth of ESCC. **a** WST-1 assay showed cell growth after 48 h of culture. **b** Trypan blue assay showed the viable cell number of ESCC cells with several inhibitors or IGF-IR/482st. **c** Soft agar assays detected that ad-IGF-IR/dns blocked colony formation. **d**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

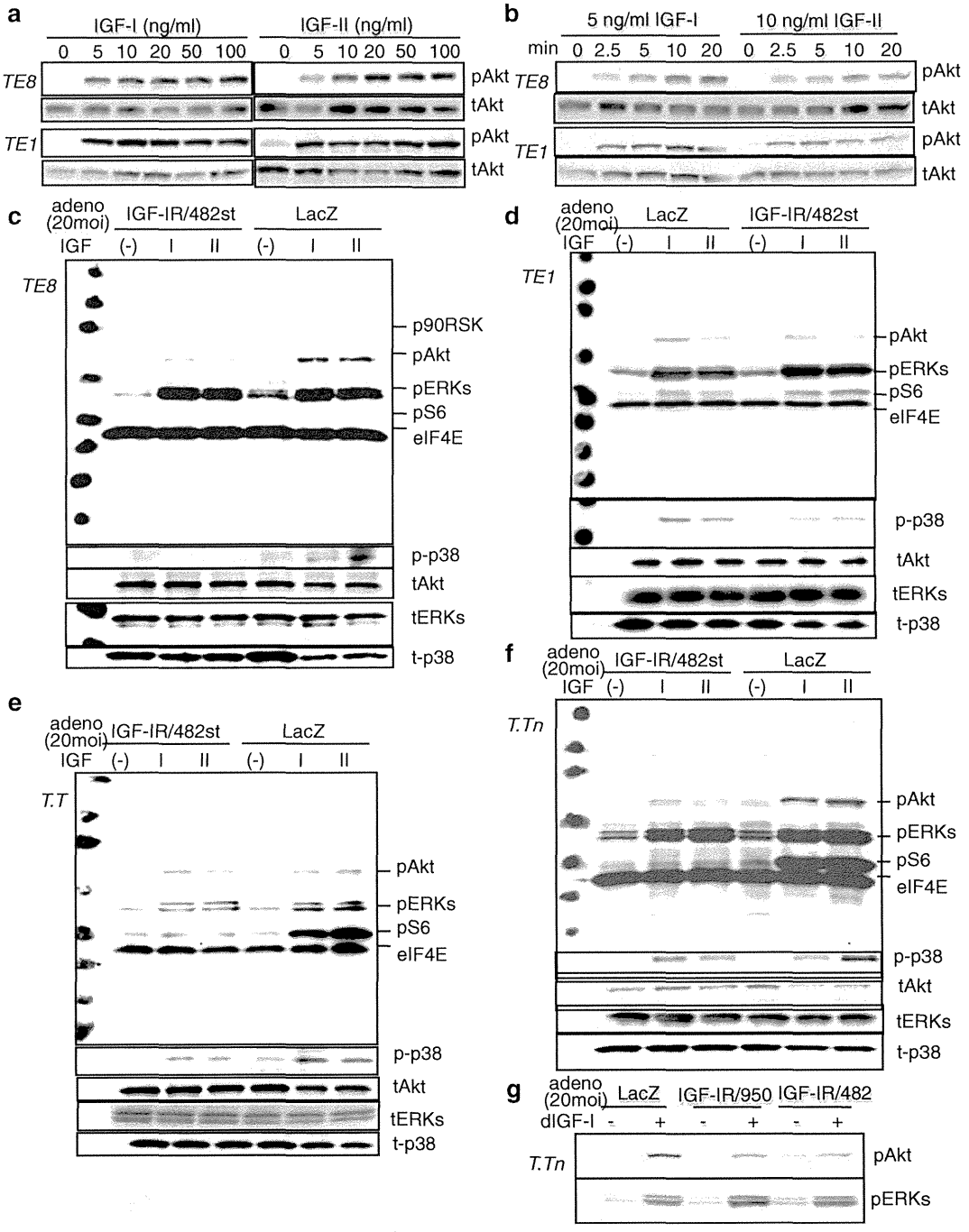

Fig. 4 The down-stream signals from IGF-IR by Western blotting. **a** Both TE8 and TE1 cells were stimulated for 5 min with IGFs, and then whole cell lysates were extracted. **b** Both cells were stimulated from 0 to 20 min with IGFs. **c**–**f** Four cell lines infected with adenoviruses were stimulated 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