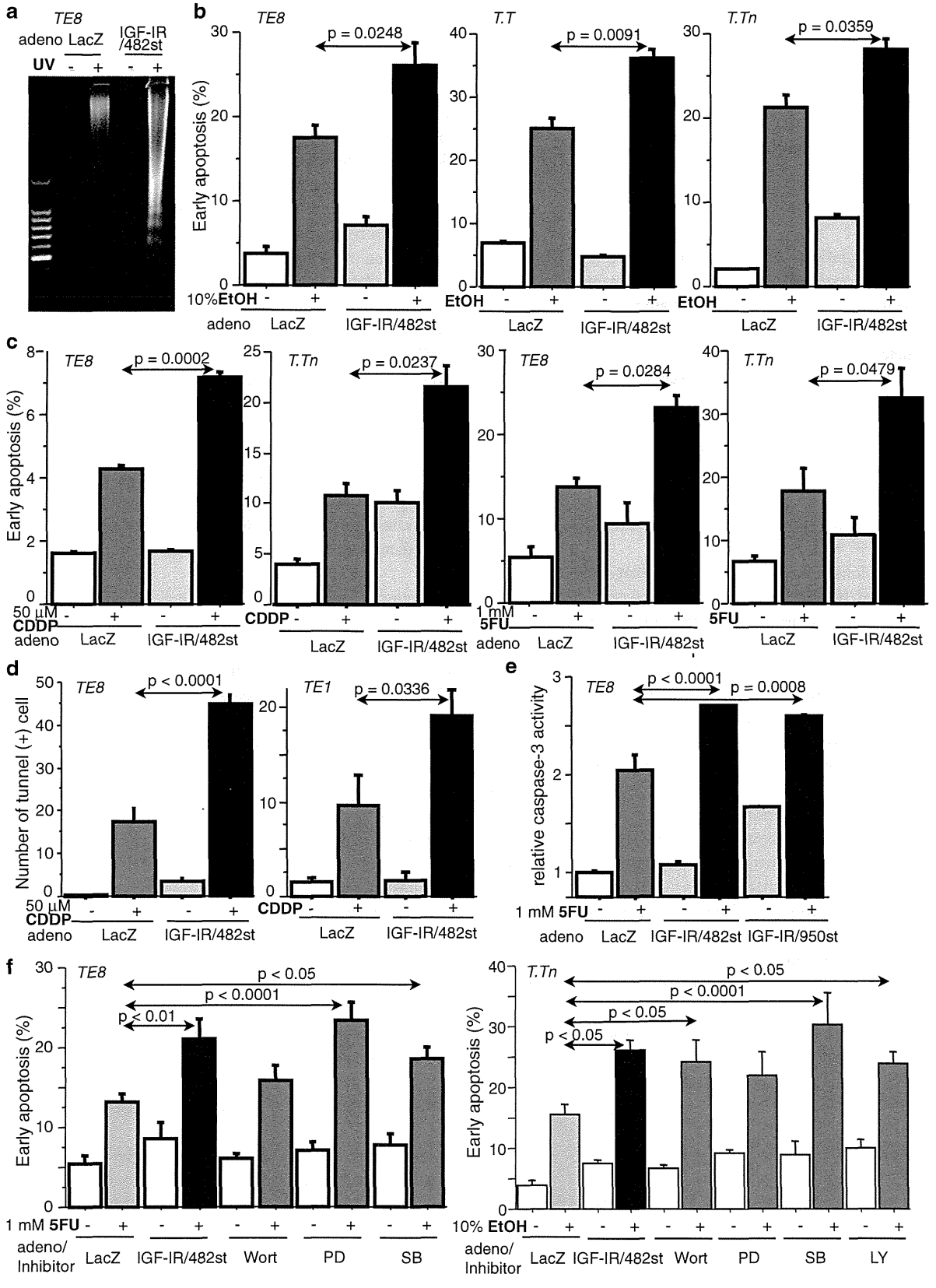

◀ **Fig. 5** Apoptotic induction in ESCC. **a** DNA fragmentation assay showed UV-induced apoptosis. **b** Annexin-V assay revealed ethanol (EtOH)-induced early apoptosis. **c** Cells were treated with chemotherapy for 24 h. Then, annexin-V assay detected early apoptosis. **d** TUNEL assay revealed cisplatin-induced 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

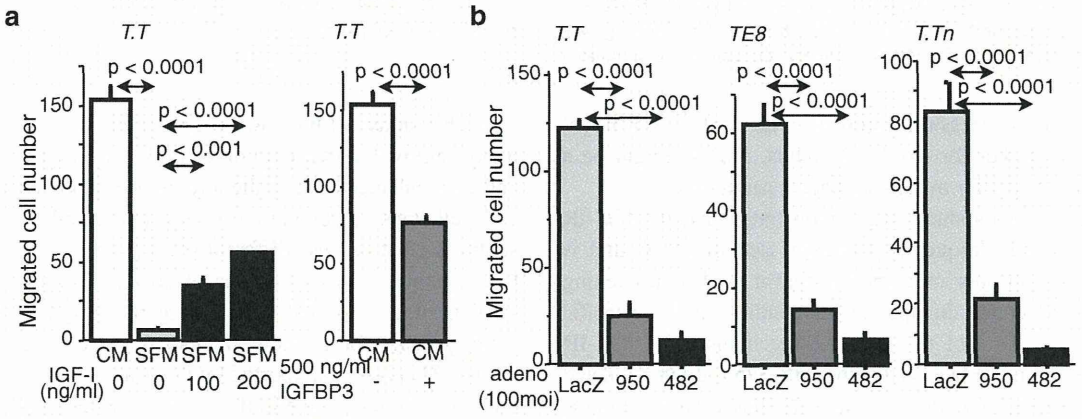

Fig. 6 The effect of IGF axis on migration of ESCC assessed by wounding assays. **a** TT cells were cultured with or without FBS ± IGF-I for 24 h and were cultured with/without IGFBP3. **b** Migration assay was performed for adenoviruses-infected cells

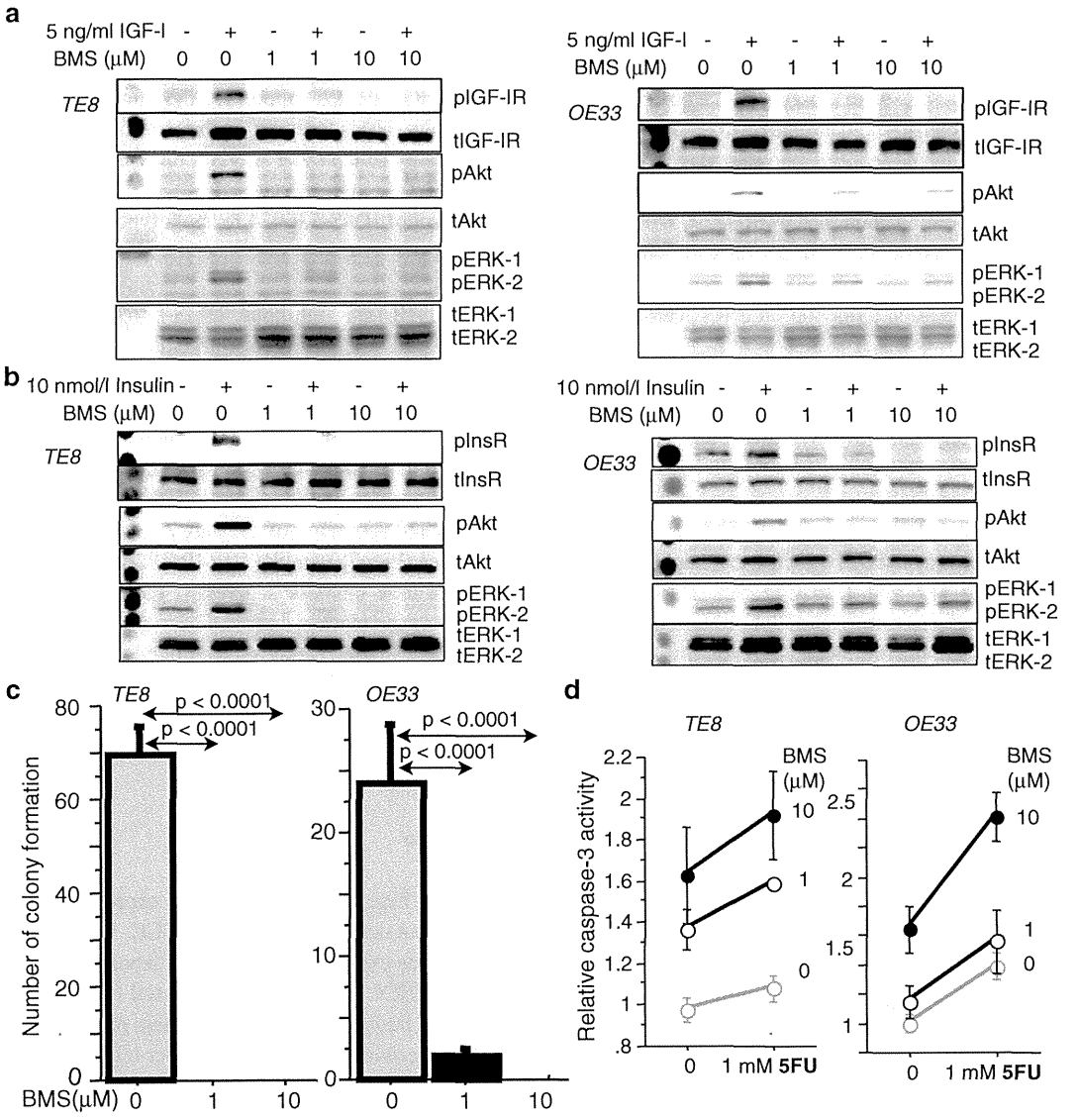

Fig. 7 The effect of BMS-536924 on both ESCC, TE8 and EAC, OE33. **a** After the cell was cultured with several amounts of BMS-536924, cells were stimulated for 5 min with IGF-I. Then Western blotting was performed. **b** After BMS-536924 treatment, the cells were stimulated for 5 min with insulin. **c** 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.

Acknowledgments This work was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology and from the Ministry of Health, Labor and Welfare, Japan. This work was also supported by a grant from the Japanese Society of Strategies for Cancer Research and Therapy.

Conflicts of interest None

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World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i14.3927

World J Gastroenterol 2014 April 14; 20(14): 3927-3937
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

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*

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Supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan
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Received: October 28, 2013 Revised: January 13, 2014
Accepted: March 7, 2014
Published online: April 14, 2014

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

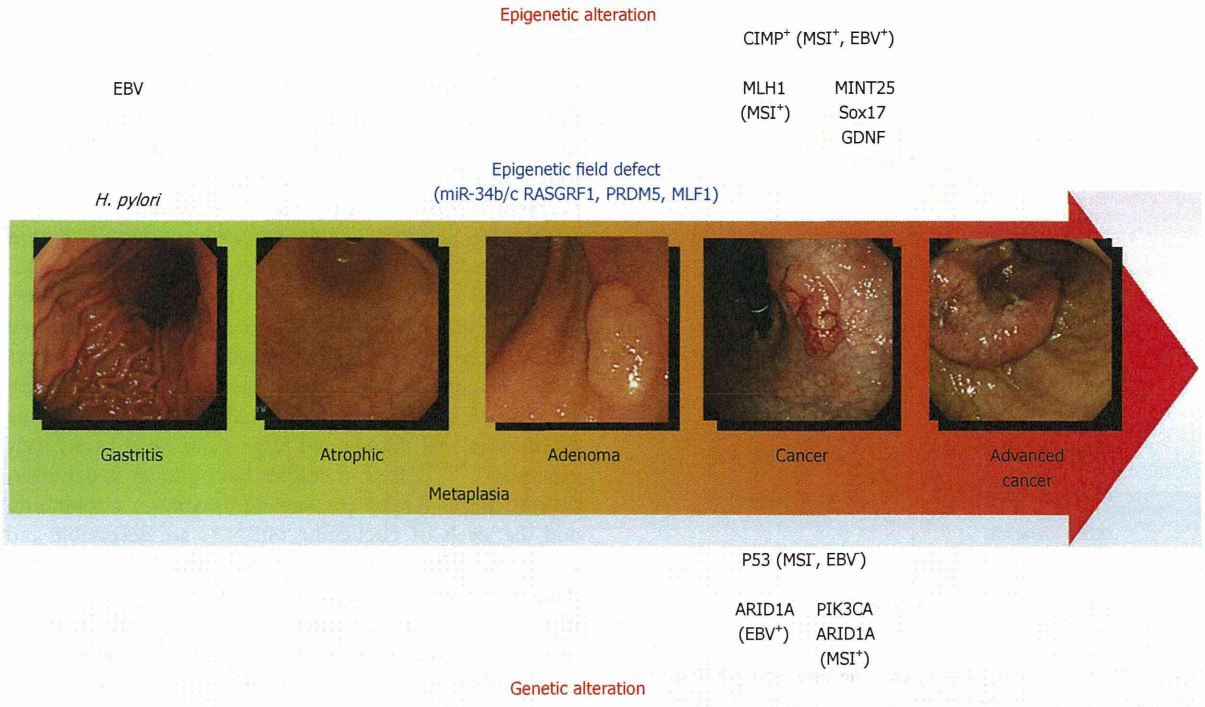

Figure 1 Genetic and epigenetic alterations in gastric carcinogenesis. The model for gastric carcinogenesis is presented based on genetic and epigenetic alterations. Methylation of the genes in blue appears to be involved in an epigenetic field defect. *H. pylori*: *Helicobacter pylori*; MSI: Microsatellite 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,

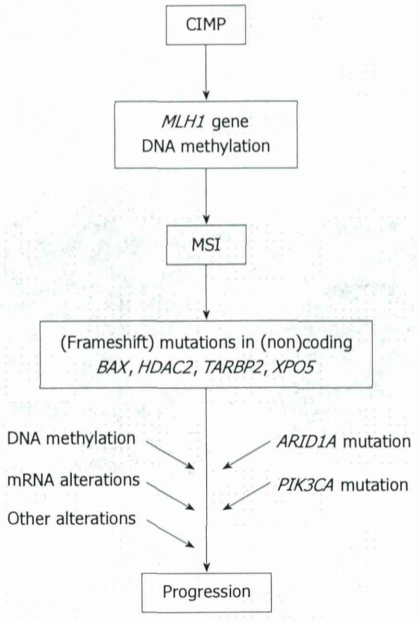

Figure 2 Molecular pathway for microsatellite instability+ gastric cancer. The model for the carcinogenesis of microsatellite instability (MSI)$^+$ gastric cancer is presented. CIMP: CpG island methylator phenotype.

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-