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Clinicopathological Significance of Aberrant Expression of IER5 Protein in Alimentary Tract Cancers

Jie Du[1†], Quanxu Wang [1†], Xiaodan Liu[2], Kuke Ding [3], Xiulian Cheng[4], Pingkun Zhou [2] and Chuanjie Yang [1*]

[1]Department of Gastroenterology, the Second Hospital of Hebei Medical University; Hebei Key Laboratory of Gastroenterology, Hebei Institute of Gastroenterology, Hebei Clinical Research Center for Digestive Disease, Shijiazhuang city, Hebei Province, China
[2]Department of Radiation Toxicology and Oncology, Beijing Institute of Radiation Medicine, Beijing, China
[3]National Institute for Radiological Protection, Chinese Center for Disease Control and Prevention and Key Laboratory of Radiological Protection and Nuclear Emergency, Beijing, China
[4]Department of Gastroenterology, The First Hospital of Zhangjiakou City, Zhangjiakou City, Hebei Province, China

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ABSTRACT This study investigates the expression and clinical relevance of the immediate early response 5 (IER5) protein in various gastrointestinal cancers, including esophageal squamous cell carcinoma, gastric adenocarcinoma, colorectal adenocarcinoma and hepatocellular carcinoma. Western blot and immunohistochemistry analyses were conducted to compare the IER5 protein levels in tumour tissues with adjacent normal tissues. The results indicate that IER5 protein expression is significantly higher in tumour tissues across all the examined cancer types, and the increase in cytoplasmic IER5 expression in these cancers suggests its potential involvement in tumorigenesis. However, this study's clinicopathological analysis did not find a correlation between IER5 expression and patients' age, sex, clinical stage and lymph node metastasis status in all the examined cancer types, as well as HBsAg positivity and cirrhosis in HCC. In conclusion, IER5 is overexpressed in gastrointestinal tumour tissues and could be a promising marker and target for cancer diagnosis and treatment.

INTRODUCTION

Oncogenesis is usually closely associated with the altered expression of certain genes. Immediate early response (IER) genes mediate transcription control upon extracellular signalling. A range of external stimuli, including growth factors, hormones or stress, upregulate IER genes rapidly. Among the ~100 IER genes, FOS has been intensively studied, it is known that histone H3K14 (H3 Lys^{14}) acetylation and H3S10 (H3 Ser^{10}) phosphorylation activate the FOS gene, which plays an important role in cancer invasion (Lee et al. 2013; Yoshida and Ibuki 2014).

The IER5 gene belongs to a slow early response gene family. The human IER5 gene is located on chromosome 1, 1q25.3 (Wan et al. 2004; Williams et al. 1999) and is expressed in tumour and normal tissue. It controls G2/M transition and is vital for cell growth and apoptosis regulation (Jansova et al. 2006; Kis et al. 2006; Okada et al. 2005). It has been reported that the IER5 gene responds to radiation (Kis et al. 2006), and previous studies have shown that IER5 is radiation inducible in a variety of cancer cells (hepatoma HepG2, cervical cancer Hela, breast cancer MCF-3, lung cancer A549, brain BT125), with the highest expression in liver cancer cells (Ding et al. 2009; Wang et al. 2006). Moreover, enhanced IER5 gene expression suppressed the growth of Hela and HepG2 cells while the known-

*Address for correspondence:
Chuanjie Yang
Department of Gastroenterology,
The Second hospital of Hebei Medical University,
Hebei Key Laboratory of Gastroenterology,
Hebei Institute of Gastroenterology,
Hebei Clinical Research Center for Digestive Disease,
NO.215.Heping West Road,
Shijiazhuang 050000, Hebei, China. .
Telephone: +86 13931100717
E-mail: 13931100717@163.com

down of the IER5 gene promoted the proliferation of the tumour cells. As such, the researchers believe that IER5 functions as a tumour suppressor gene. In fact, a recent study on acute myeloid leukaemia suggested that IER5 inhibits tumour cell proliferation by suppressing the cell division cycle 25B (Cdc25B) gene expression through directly binding to Cdc25B promoter with nuclear transcription factor Y, subunit B (NF-YB) and p300 transcriptional factors (Nakamura et al. 2011). However, the expression of the IER5 gene in other tumours is vastly unknown. Besides, the existence of hepatitis B surface antigen (HBsAg) in hepatitis B virus infection was associated with liver cirrhosis (Wang et al. 2022), while serum HBsAg levels can predict liver fibrosis in HBeAg-positive hepatitis B cirrhosis (Shi et al. 2022).

The objective of this study is to examine the expression and role of IER5 in gastrointestinal cancers, including esophageal squamous cell carcinoma, gastric carcinoma, hepatocellular carcinoma (HCC) and colorectal carcinoma (CRC), and its correlation with clinical features, using Western blot and immunohistochemistry (IHC) to determine its potential as a marker for cancer diagnosis and therapeutic target. The researchers found that IER5 was differentially expressed in both tumour cells and para-tumor normal parenchymal cells. The researchers also demonstrated that this enhanced IER5 gene expression was not correlated with clinical features in all four cancers. The results indicate that IER5 is involved in the development of gastrointestinal cancers.

MATERIAL AND METHODS

Study Design and Participants

A total of 10 cases for each of the four cancers (esophageal squamous cell carcinoma, gastric carcinoma, HCC and CRC) were enrolled for this study from October 2010 to January 2011 at the researchers' hospital, a tertiary medical centre. The 40 patients included 25 males and 15 females. The average ages of patients with esophageal squamous cell carcinoma, gastric carcinoma, HCC and CRC were 55-, 56-, 50- and 61-year old, respectively. Detailed clinical and demographic data were collected to analyse the correlation between IER5 expression and various clinicopathological parameters, and the inclusion of a range of ages across the cancers under study allows for a comprehensive examination of IER5 expression across a diverse patient group. Informed consent forms were signed and obtained. The study was approved by the Ethics Committee of the institution.

Specimen Collection and Processing

No radiation or chemotherapy was given to all the patients. A total of 10 surgical resection specimens for each cancer were obtained. Tumour tissues and para-tumour normal tissues 5 cm from surgical margins were collected within 30 minutes of the removal of tumours. Tumour necrosis was identified pathologically post-surgery. The specimens were stored in liquid nitrogen for further Western blot analysis or in 4 percent formalin for further IHC study to determine the expression levels of the IER5 protein in the investigated specimens.

Protein Extraction and Western Blot Analysis

About 100 mg of tissue was washed with cold PBS, minced and treated with a RIPA buffer containing 1mM PMSF. The tissue was homogenised, lyzed on ice for 10 minutes, and spun at 4^0C, 14,000g × 15 minutes. The supernatant was assessed for protein concentration by the BCA method, aliquoted and stored at -80°C. A total of 100μg of protein was loaded onto each lane of SDS-PAGE gel. IER5 protein was detected by Western blot using rabbit anti-human IER5 antibody (Sigma, USA). Beta-actin was used as the loading control. The exposed film was scanned using an imaging analysis system. Densities of the IER5 band and β-actin were determined, and the relative expression level of IER5 protein was presented as the ratio of density of the IER5 band/density of β-actin band for better comparison of IER5 expression levels between tumour and adjacent normal tissues.

IHC Procedures

The IHC section details the procedure for visualising the localisation and expression pattern of the IER5 protein within the tissue samples. To examine the expression and localisation of IER5 in the four cancers and para-tumor nor-

mal tissues, the researchers also did IHC staining on the 4 percent formalin-fixed tissues using rabbit anti-human IER5 antibody (Sigma, USA). The average optic density of tumour cells in 10 high power fields (200×) was measured using the Motic Med Digital Medical Imaging System.

Statistical Analysis

The band intensity of WB was measured using Image J software. The change in protein expression level was determined by the band intensity ratio between tumour and para-tumor samples. Continuous quantitative data was presented as mean±SD. The differences between the two groups were compared using paired T-test. Comparison between multiple groups was done with univariate analysis of variance (one-way ANOVA). Rank data between groups were compared using LSD and SNK tests. Statistical analysis was done with SPSS 13.0 software. P <0.05 was considered statistically significant.

RESULTS

Enhanced Expression of IER5 in Gastrointestinal Cancers

The researchers first employed the Western blot method to detect expression of IER5 in human gastrointestinal cancers, namely esophageal squamous cell carcinoma, gastric adenocarcinoma, CRC and HCC, with comparison to that in para-tumour normal tissues. Ten cases were examined for each tumour, and total cellular proteins were probed with the IER5 antibody. The results demonstrated that in each case, including the tumour tissue and the normal tissue, all showed a single reactive band at the position of a molecular weight of 32 kilodaltons, which was consistent with the pattern appearance of the antibody. The results indicated that the antibody specifically recognises IER5 proteins in the cell lysates.

The relative expression of IER5 protein was calculated as the density to that of the corresponding normal tissues. Specifically, compared to that of the corresponding normal tissues, IER5 expression levels in esophageal squamous cell carcinoma, gastric cancer, HCC and CRC were upregulated by 148 percent, 71 percent, 172 percent and 96 percent ($p < 0.05$, Fig. 1). Taken together, these results demonstrated that IER5 expression is overexpressed in all the four investigated cancer types.

Overexpression of IER5 in Gastrointestinal Cancers and Lack of Correlation with Clinicopathological Features

To ensure representative tissues were sampled, the researchers also performed H&E staining on all 40 surgical specimens. Pathological evaluation showed that all tumour tissues were viable without necrosis, and all corresponding normal tissues were not invaded (Figs. 2-5).

To further look into the significance of the enhanced IER5 in these 4 cancers, the researchers investigated the correlation of expression levels of IER5 with clinicopathological features of these tumours by a correlation analysis. The data showed that IER5 protein expression in esophageal squamous cell carcinoma, gastric adenocarcinoma, HCC and CRC was not correlated with the patients' age, gender, clinical stage and status of lymph node metastasis (p all > 0.05). Since 70-80 percent of HCC cases in China have a background of hepatitis B virus infection and/or concomitant cirrhosis. In HCC, the researchers also specifically studied the correlation of IER5 protein expression levels with HBsAg positivity and the existence of cirrhosis. However, it turned out that IER5 protein expression levels were not different between the patients with different statuses of HBsAg infection and cirrhosis (p all> 0.05) (Tables S1-S4). These results suggested that IER5 could be a tumour marker for gastrointestinal cancers playing a role in the development of the neoplasms instead of tumour progression.

The Western blot analysis used reflected the expression of IER5 in all tumour cells. To visualise intracellular expression and localisation of IER5 protein, the researchers further immunohistochemically observed intracellular IER5 proteins by using the same anti-IER5 antibody used above. Analysis of the immunohistochemical images revealed that all the tumour tissues and their corresponding normal tissues were positive for reaction to IER5, presenting as typical cytoplasm brown staining (Figs. 2-5). In regard to the amount of IER5 expressed by the tumours,

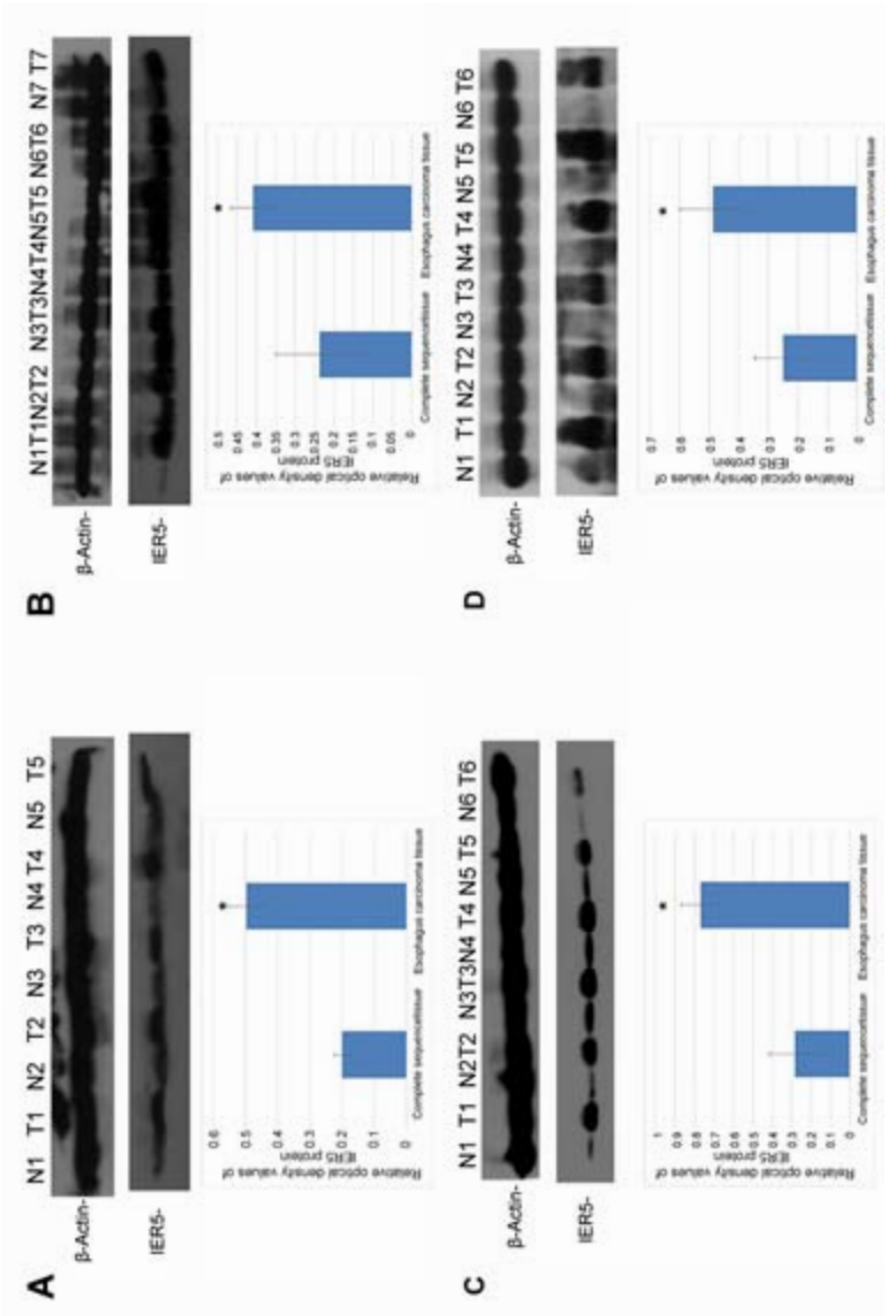

Fig. 1. Expression of IER5 protein in human esophageal, gastric cancer, HCC and CRC. Western-blot analysis with whole tumor cell lysates from 5 cases of human esophageal (A), seven cases of gastric cancer (B), six cases of HCC (C) and six cases of CRC (D) were shown. Relative expression of the IER5 gene relative to that of ?-actin was plotted below the corresponding western-blot Fig. N: para-tumor normal tissue; T: carcinoma tissues; *: P<0.05

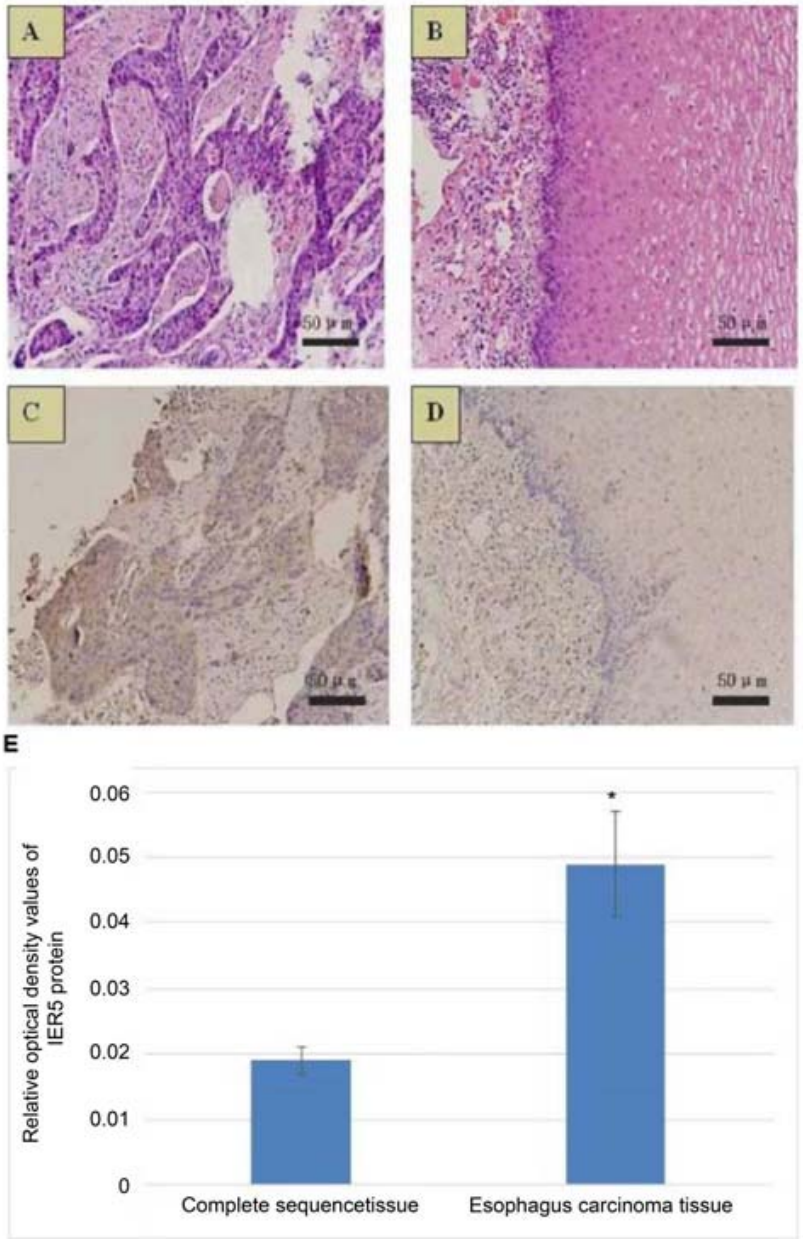

Fig. 2. Expression of IER5 protein in human esophageal cancer detected by IHC (×200). A, histological images of esophageal carcinoma tissues with H&E staining. B, histological images of complete sequence tissues with H&E staining. C, immunoreactivity of esophageal carcinoma to IER5 antibody. D, immunoreactivity of para-tumor normal tissue to IER5 antibody. E, optic densities of IER5 positivity in the cancer tissue and the para-tumor normal tissue
***: P<0.05**

Table S1 The relationship between IER5 protein in Esophagus carcinoma and clinical characteristics

Characteristics	*No. of cases*	*Western-blot analysis*	*p-values*	*Immunohistochemistry analysis*	*p-values*
Gender			*0.202*		*0.125*
Male[#]	6	0.407±0.056		0.052±0.002	
Female[#]	4	0.408±0.057		0.051±0.003	
Age (years)			*0.202*		*0.124*
<50[#]	4	0.406±0.055		0.049±0.002	
>50[#]	6	0.409±0.058		0.053±0.002	
TNM staging			*0.201*		*0.125*
I, II[#]	3	0.405±0.056		0.053±0.002	
III, IV[#]	7	0.408±0.057		0.051±0.003	
Lymph Node Metastasis			*0.202*		*0.125*
No[#]	4	0.407±0.054		0.052±0.002	
Yes[#]	6	0.408±0.056		0.051±0.003	

Note: [#]p>0.05

Table S2: The relationship between IER5 protein in gastric carcinoma and clinical characteristics

Characteristics	*No. of cases*	*Western-blot analysis*	*p-values*	*Immunohistochemistry analysis*	*p-values*
Gender			*0.202*		*0.125*
Male[#]	6	0.407±0.056		0.052±0.002	
Female[#]	4	0.408±0.057		0.051±0.003	
Age (years)			*0.202*		*0.124*
<50[#]	4	0.406±0.055		0.049±0.002	
>50[#]	6	0.409±0.058		0.053±0.002	
TNM staging			*0.201*		*0.125*
I, II[#]	3	0.405±0.056		0.053±0.002	
III, IV[#]	7	0.408±0.057		0.051±0.003	
Lymph Node Metastasis			*0.202*		*0.125*
No[#]	4	0.407±0.054		0.052±0.002	
Yes[#]	6	0.408±0.056		0.051±0.003	

Note: [#]p>0.05

esophageal squamous cell carcinoma, gastric cancer, HCC and CRC tumour cells all produced more IER5 proteins in the cytoplasm with the magnitude of increase of 157 percent, 63 percent, 83 percent and 72 percent respectively ($p < 0.05$), comparing to those in the corresponding adjacent normal cancer. In addition, the clinical and pathological analysis showed similar significance of this increase in expression with that observed in Western blot evaluation, while cytoplasmic IER5 expression was not associated with changes of age, sex, clinical stage and status of lymph node metastasis (p all> 0.05). In HCC, consistently, IER5 was not associated with HBsAg positivity or status of cirrhosis (p all > 0.05). Collectively, these findings indicate that IER5 expression is significantly increased in various gastrointestinal cancers but not linked to patient demographics or disease progression, suggesting its role in cancer development rather than advancement.

Variability of IER5 Expression Among Different Tumours

Another interesting finding of this study was that IER5 expression varied significantly among these four tumours (Fig. 6), potentially indicating differential degrees of impact in various gastrointestinal cancers. The Western blot results showed that the relative expression levels of IER5 protein in these four tumours lined up in descending order as HCC> esophageal squamous cell carcinoma> CRC> gastric adenocarcinoma ($p < 0.05$), whereas IER5 expression levels demonstrated on IHC differed among the tumours in

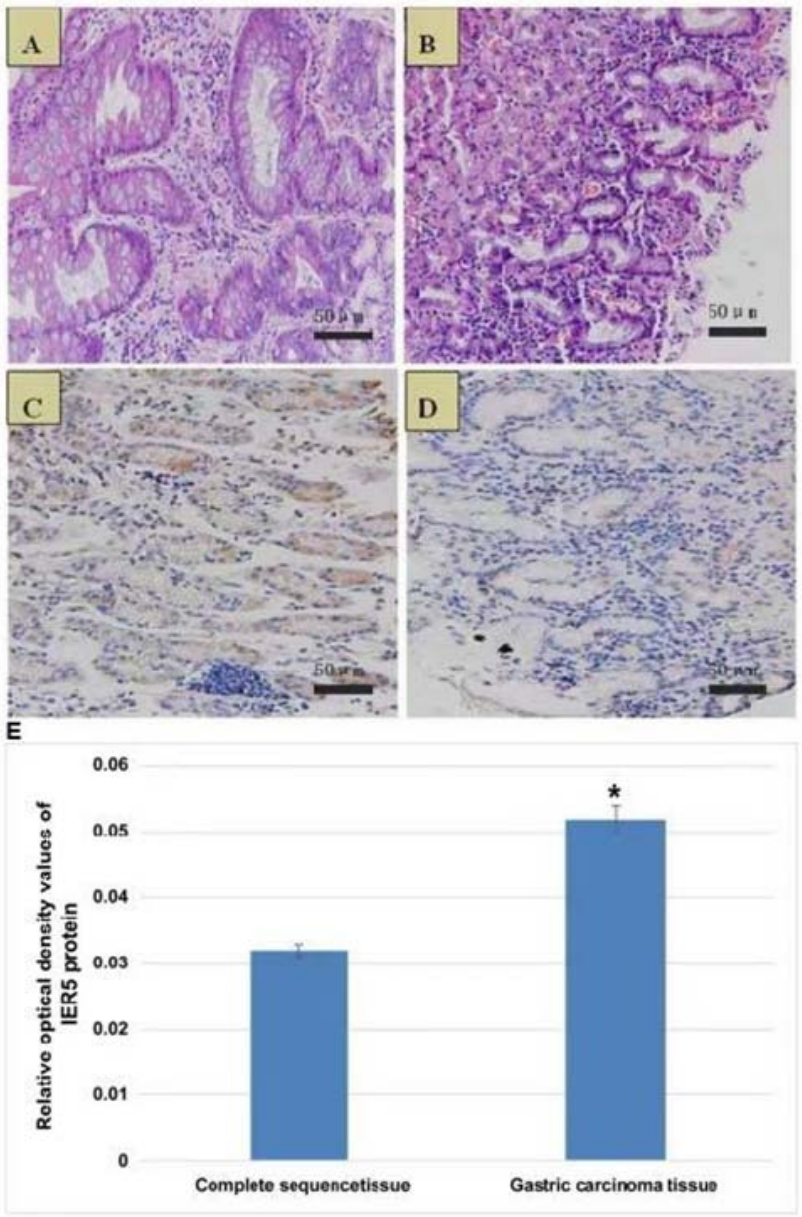

Fig. 3. Expression of IER5 protein in human gastric cancer detected by IHC (×200). A, histological images of gastric carcinoma tissues with H&E staining. B, histological images of complete sequence tissues with H&E staining. C, immunoreactivity of gastric carcinoma to IER5 antibody. D, immunoreactivity of para-tumor normal tissue to IER5 antibody. E, optic densities of IER5 positivity in the cancer tissue and the para-tumor normal tissue.
***: P<0.05**

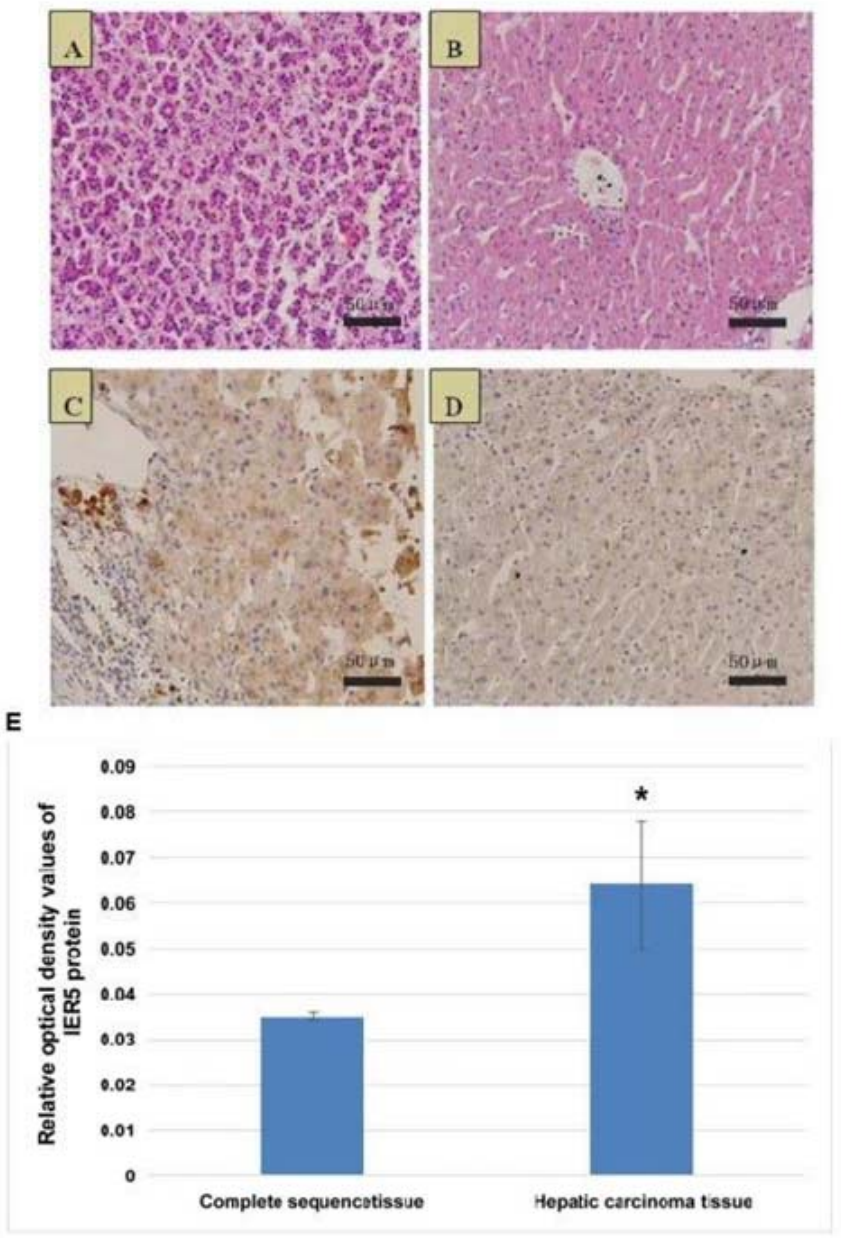

Fig. 4. Expression of IER5 protein in human HCC detected by IHC (×200). A, histological images of HCC tissues with H&E staining. B, histological images of complete sequence tissues with H&E staining. C, immunoreactivity of HCC to IER5 antibody. D, immunoreactivity of para-tumor normal tissue to IER5 antibody. E, optic densities of IER5 positivity in the cancer tissue and the para-tumor normal tissue. *: P<0.05

Table S3: The relationship between IER5 protein in hepatocellular carcinoma and clinical characteristics

Characteristics	*No. of cases*	*Western-blot analysis*	*p-values*	*Immunohistochemistry analysis*	*p-values*
Gender			*0.301*		*0.301*
Male#	8	0.769±0.101		0.057±0.009	
Female#	2	0.767±0.100		0.055±0.008	
Age (years)			*0.300*		*0.300*
<50#	6	0.768±0.100		0.056±0.008	
>50#	4	0.769±0. 009		0.057±0.008	
Size (cm)			*0.301*		*0.301*
d”5#	4	0.765±0.101		0.057±0.009	
>5	6	0.768±0.100		0.056±0.008	
HbsAg (ng/ml)			*0.302*		*0.302*
Absent#	2	0.764±0.101		0.055±0.008	
Present#	8	0.770±0.101		0.059±0.008	
Cirrhosis			*0.301*		*0.301*
No#	3	0.767±0.100		0.055±0.009	
Yes#	7	0.769±0.100		0.057±0.009	
TNM staging			*0.301*		*0.301*
I	3	0.766±0.101		0.056±0.007	
II-III	7	0.768±0.101		0.059±0.008	

Note: # p>0.05

Table S4: The relationship between IER5 protein in Colorectal carcinoma and clinical characteristics

Characteristics	*No. of cases*	*Western-blot analysis*	*p-values*	*Immunohistochemistry analysis*	*p-values*
Gender			*0.220*		*0.124*
Male#	5	0.487±0.112		0.064±0.014	
Female#	5	0.486±0.111		0.063±0.013	
Age (years)			*0.219*		*0.124*
<50#	3	0.485±0.111		0.062±0.013	
>50#	7	0.488±0.111		0.064±0.013	
TNM Staging			*0.220*		*0.124*
I, II#	4	0.486±0.112		0.060±0.012	
III, IV#	6	0.489±0.111		0.063±0.013	
Lymph Node Metastasis			*0.220*		*0.124*
No#	4	0.486±0.113		0.060±0.012	
Yes#	6	0.489±0.110		0.061±0.012	

Note: # p>0.05

descending order as HCC> colorectal cancer> stomach cancer> esophageal squamous cell carcinoma (p <0.05, Tables S1-S4).

DISCUSSION

Oncogenesis is closely regulated by certain genes (Banno et al. 2012; Jiang and Chen 2012; Leber and Efferth 2009; Li et al. 2015; Liao et al. 2012; Mizuguchi et al. 2015; Yusoff 2015; Peng et al. 2014; Pierard and Pierard-Franchimont 2012; Sianou et al. 2015; Sui et al. 2013). Abnormalities of gene expression are very common in digestive tract tumours. For example, the Wnt/â-catenin signalling pathway plays a vital role in the initiation of sporadic colorectal tumours, and β-catenin is a key regulator of colorectal cancer (Mojarad et al. 2015; Yu et al. 2015). The IER5 gene (immediate early response 5) is a family member of the slow early response genes regulating physiological function and pathogenesis in a variety of conditions (Ding et al. 2009; Ishikawa and Sakurai 2015; Li et al. 2011; Nakamura et al. 2011; Okada et al. 2005; Tavakoli et al. 2013; Wil-

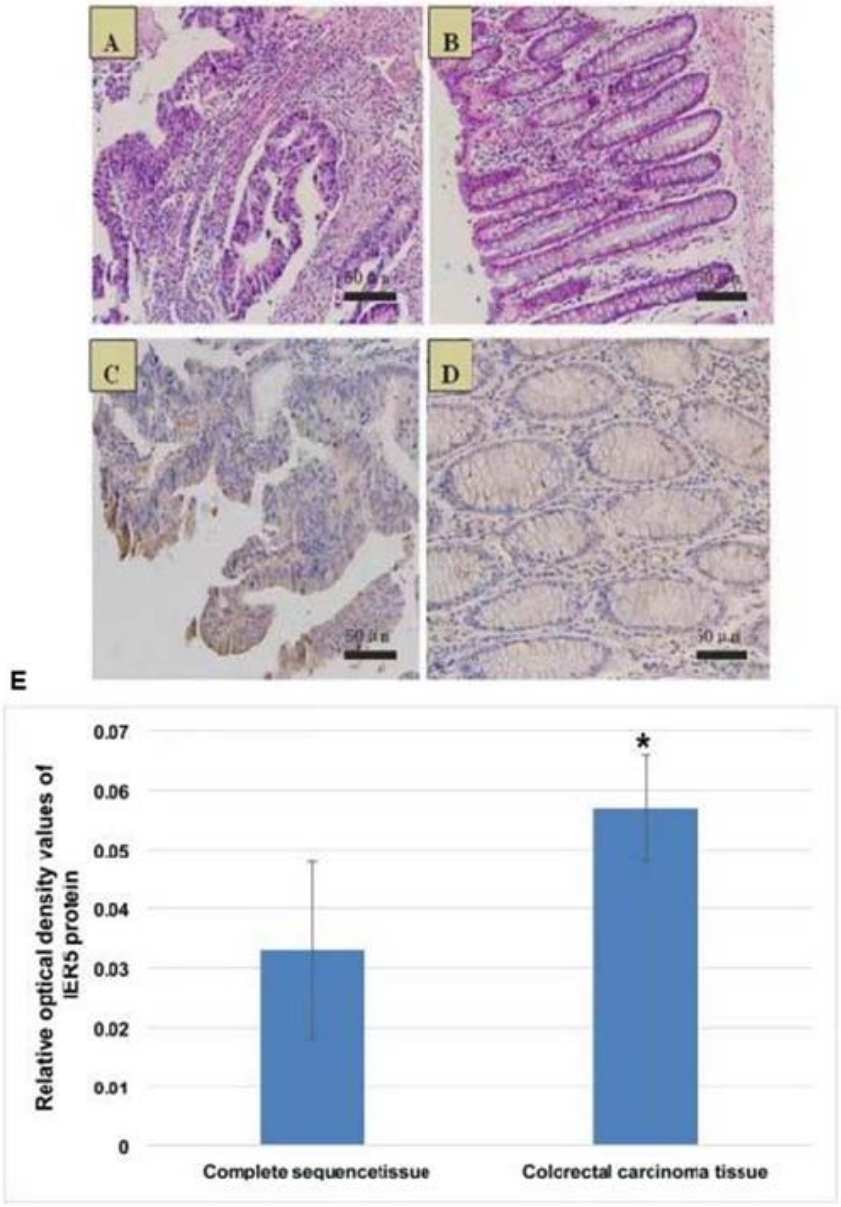

Fig. 5. Expression of IER5 protein in human CRC detected by IHC (×200). A, histological images of CRC tissues with H&E staining. B, histological images of complete sequence tissues with H&E staining. C, immunoreactivity of CRC to IER5 antibody. D, immunoreactivity of para-tumor normal tissue to IER5 antibody. E, optic densities of IER5 positivity in the cancer tissue and the para-tumor normal tissue
***: P<0.05**

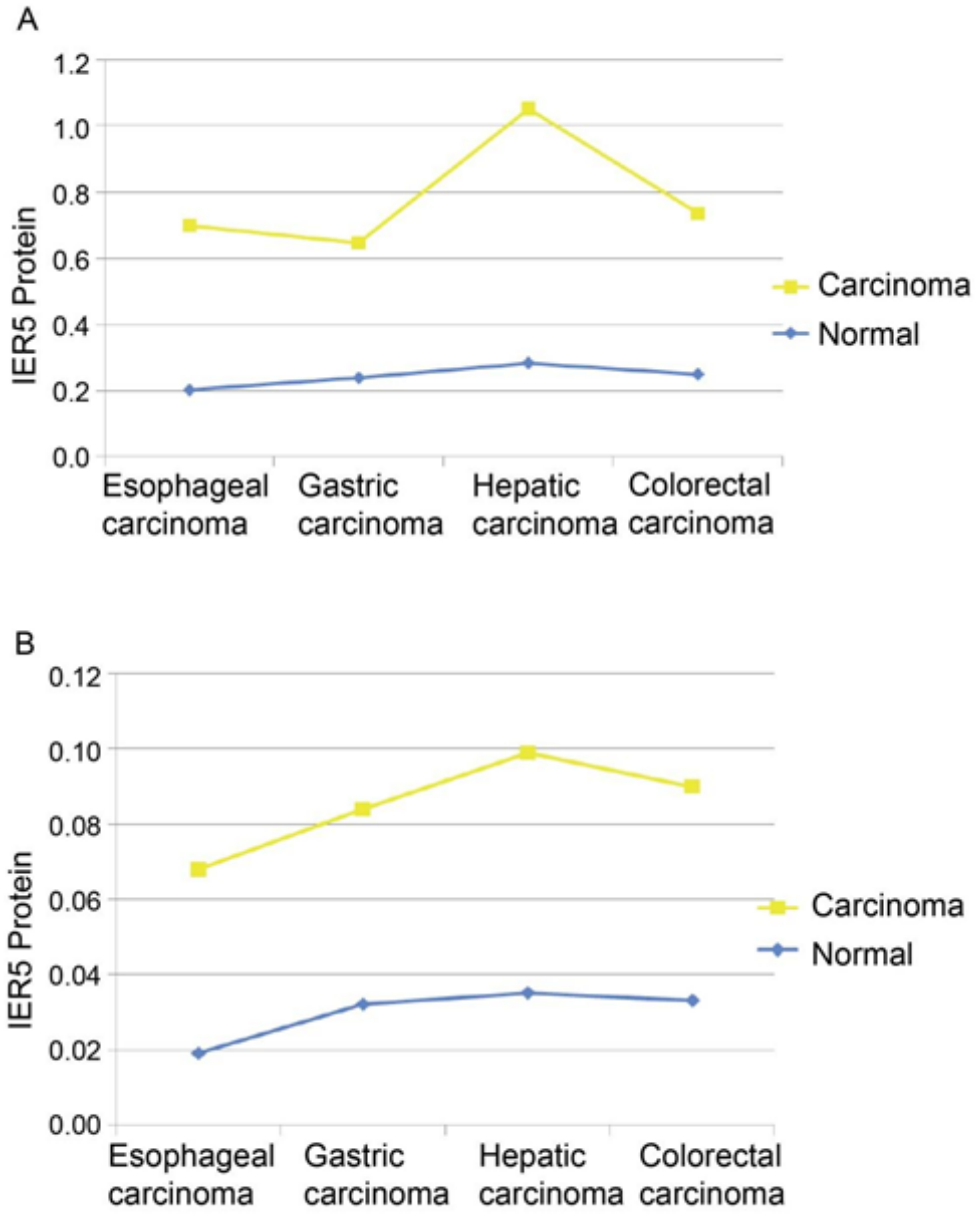

Fig. 6A. Differences of the IER5 protein expressions among digestive systematic carcinomas by western-blot method; B: Differences of the IER5 protein expressions among digestive systematic carcinomas by immunohistochemical SP method. The IER5 protein levels of hepatic carcinoma were highest than the other (P<0.05)

liams et al. 1999). The researchers have previously found that the IER5 gene plays a role as a tumour suppressor gene in the development of liver cancer and cervical cancer and response to radiation. Using tumour cell lines, the researchers identified more than 30 genes that were upregulated upon radiation, including the IER5 gene. The researchers further confirmed that IER5 was responsible for the radiosensitivity of HCC in vivo and behaved as a tumour suppressor gene (Ding et al. 2009; Wang et al. 2006; Zhou and Rigaud 2001). In the present study, the researchers found that with respect to adjacent normal tissues, IER5 expression in esophageal cancer, gastric cancer, HCC and CRC was significantly enhanced. However, this change did not correlate with the patients' clinical features in any of these four cancers. Overall, these suggest that IER5 plays a significant tumour suppressor role in various gastrointestinal cancers, with enhanced expression not correlating with clinical features, and could be a potential marker for cancer detection and a target for cancer treatment.

It is widely acknowledged that the development of tumours is a consequence of abnormal signal transduction involving a range of pathways, which are composed of imbalanced expression of oncogenes and tumour suppressor genes, disturbed stimuli of growth factor and matrix metalloproteinases, etc. (Houtgraaf et al. 2006; Ji et al. 2014; Leber and Efferth 2009; Matyszewski et al. 2015; Zheng et al. 2013). Targeting these molecular events provides a possibility of managing the cancers molecularly. Factually, advances in molecular therapy in recent years have greatly improved the prognosis of cancers (Russo et al. 2015; de Ronde et al. 2014; Fava et al. 2015; Kalia 2015).

The IER5 gene is located on chromosome 1, has 2123 encoding nucleotides but no introns, and possesses homology with a panel of early reaction genes, including p92, IER2, and ETR101 at the amino terminus (Gregory et al. 2006). Studies have shown that IER5 regulates normal physiological activities. For example, IER5 mediates the response to oestrogen in the endometrium (Wu et al. 2003), regulates development, sleep, arousal, and hematopoiesis (Cirelli and Tononi 2000; Matsuo et al. 2000; Tononi and Cirelli 2001). IER5 is also tumour-related, and it modulates cellular response to mitogenic signals. Colon cancer patients harbour mutations of IER5, among other genes (Kis et al. 2006; Wan et al. 2004). These highlight the potential role of IER5 in both normal bodily functions and cancer development. Its involvement in regulating responses to hormonal signals and cellular growth suggests IER5 could be a significant target for therapeutic interventions in cancers, particularly where its mutation contributes to oncogenesis.

Digestive cancers are common tumours, accounting for 60 to 70 percent of all malignancies. Moreover, increasing incidences of gastric cancer, liver cancer, esophageal cancer and colorectal cancer has been noted in recent years in different populations, especially with a trend of increase in young people (Bergquist and von Seth 2015; Pourhoseingholi et al. 2015; Puleo et al. 2015; Singal and El-Serag 2015; Song et al. 2015; Tsuei et al. 2014; Zhang et al. 2014). The role of the IER5 gene in the development of human digestive system tumours is not fully clear yet. Based on the researchers' previous and present findings, they speculate that the cells depolarise upon various stimuli, leading to activation of the dormant IER5, which consequently regulates the repair process, cell proliferation, growth and differentiation. The universal upregulated but not stage-related IER5 expression in the four digestive tract cancers found in this study indirectly confirmed the hypothesis. The findings suggest that the loss of IER5 function as a tumour suppressor gene might initiate the cancerogenesis of these cancers.

The present study also showed that IER5 protein was aberrantly expressed among esophageal squamous cell carcinoma, gastric adenocarcinoma, HCC and CRC, and Western blot and IHC showed unmatched differences in IER5 expression. This finding indicated two aspects of IER5 in digestive tract cancers. On the one hand, the IER5 gene may exert type-specific action in the development of cancer. Second, intracellular translocation may be one of the mechanisms underlying cellular regulation by IRE5. The differential expression and localisation of IER5 in various digestive tract cancers suggest it may play distinct roles in the oncogenesis of different cancer types. This variability underscores the potential for IER5 to serve as a biomarker for cancer diagnosis or prognosis and possibly as

a target for cancer-specific therapeutic strategies, highlighting the importance of understanding the molecular mechanisms of IER5's action in cancer development.

Previous works indicated that IER5 was associated with inhibition of tumour progression. However, higher IER5 expression is also reported to be correlated with poor prognosis in glioma patients, indicating the multiple role of IER5 in different types of tumours (Wu et al. 2021). The specific role of IER5 in the reported tumour model is still not clear, which warrants further investigation. The researchers did not find other IER family proteins to exert a similar role.

CONCLUSION

In summary, IER5 is overexpressed in digestive tract cancers and may play a role in the development of these cancers. Further study on the role of the IER5 gene in other gastrointestinal tumours and the mechanism involved will help understand the biological behaviour of gastrointestinal cancer better and offer wider insights into its potential as a novel molecular target for cancer diagnosis and treatment.

RECOMMENDATIONS

This study reveals the potential of the IER5 protein as a promising marker for the diagnosis and treatment of various cancers. Given its overexpression in tumour tissues and its involvement in tumorigenesis, despite the lack of correlation with patients' clinical features, it is suggested that future research focuses on exploring the mechanistic role of IER5 in cancer development, such as its interaction with other cellular pathways and its impact on cell proliferation and apoptosis. Additionally, the study highlights the necessity for developing therapeutic strategies that could target IER5 expression or function, which might offer a new avenue for cancer treatment, at least in in vivo and in vitro settings, to confirm the findings. Clinical trials aimed at evaluating the efficacy of such therapies could be beneficial as a future prospect. Lastly, considering the variation in IER5 expression among different gastrointestinal cancers, investigations into cancer-specific roles and mechanisms are recommended, which could lead to more personalised approaches in cancer management.

STATEMENT OF ETHIC

This study was approved by the ethics committee of Hebei Medical University (No. 2020R218). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from individual participants.

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DECLARATION OF INTEREST

All authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

Jie Du, Quanxu Wang and Chuanjie Yang contributed to the study's conception and design. All authors collected the data and performed the data analysis. All authors contributed to the interpretation of the data and the completion of figures and tables. All authors contributed to the drafting of the article and final approval of the submitted version.

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