Identification of the potential molecular mechanism and driving mutations in the pathogenesis of familial intestinal gastric cancer by whole exome sequencing

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Abstract. The genetic alterations in familial intestinal gastric cancer (FIGC) have not been clearly understood. Aiming to explore the molecular basis and the driving mutations underlying the pathogenesis of FIGC, we performed exome sequencing of the blood samples of the members of an extended family with FIGC. The differences in mutation patterns between family members with gastric cancer and controls were analysed and the overlapped variants were screened by comparing previously published data for blood and tumours from gastric cancer patients. The overlapped genes harbouring insertions-deletions (INDELs) and single-nucleotide variants (SNVs) were subjected to function, pathway and network analysis. The INDELs were enriched in DNA packaging and in the neurological system process related to the biological process (BP), while SNVs were closely related to cell-function-related BPs. ESR was the significant node with marked centrality in the SNV network. ERK 1/2 was the hub node in the INDEL network, interacting with EZK and IGF2R. Sequencing analysis revealed ESR1 homozygous mutations in exon 1 (216G > C) and exon 10 (2234C > T) and EZR1 heterozygous deletion of 68-69 GT nucleotides in exon 13 of the family members. The IGF2R gene only demonstrated a mutation in exon 48 of the propositus. All hub proteins had direct or indirect interactions in the protein-protein interaction network.

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Introduction

Gastric cancer (GC) is the second most frequent cause of cancer-related deaths worldwide (1). Several studies revealed that a positive family history of having a first-degree relative with GC is considered a strong risk factor for the development of GC, particularly when two or more relatives are affected (2). There is familial aggregation in ~10-20% of GCs and ~1-3% have a clear inherited genetic conditioning (3). A good understanding of the genetic mechanism of GC in the family may shed light on the driving genes and pathways for treatment options and genetic counselling. However, the genetic events that predispose individuals to GC have not been clearly understood.

Three hereditary GC syndromes have been described which are the following: Hereditary diffuse gastric cancer (HDGC), familial intestinal gastric cancer (FIGC) and the recently proposed gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS) (4). Some other hereditary cancer syndromes such as hereditary non-polyposis colorectal cancer (HNPCC), Li-Fraumeni syndrome (LFS), familial adenomatous polyposis (FAP) and Peutz-Jeghers syndrome (PJS) also predispose individuals to GC (5). Genetic and epigenetic alterations play key roles in the pathogenesis of familial GC development (6). Except for HDGC, the molecular basis for the familial aggregation remains largely unknown. Identification of new predisposition genes would provide novel insights regarding the molecular pathogenesis of GC. However, the pathogenesis and genetic changes of FIGC have not been clearly elucidated.

Whole-exome sequencing has been widely used to identify the genomic mutation signatures for uncovering the predisposing genes in familial cancers (7,8). In the present study, we explored three genomic variations (ESR1, IGF2R and EZR) in FIGC by whole-exome sequencing. The oestrogen receptor α/oestrogen receptor 1 (ERα/ESR1) gene, a well-known proto-oncogene, is a member of the nuclear hormone receptor family and plays an important role in hormone binding, DNA binding and activation of transcription (9). The

mannose-6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R), referred to as IGF2R, is a multifunctional protein ubiquitously expressed in human tissues and has been recently identified as a tumour suppressor (10). Ezrin, encoded by the EZR gene, is a signal transduction component belonging to the ezrin-radixin-moesin (ERM) protein family; it acts both as a link between the actin cytoskeleton and plasma membrane proteins and as a substrate for tyrosine kinase (11).

The detection of these mutations, which appear to predispose individuals to familial GC, could lead to the identification of individuals with a risk of familial GC in affected families and may be useful as biomarkers for confirmatory diagnosis of FIGC and appropriate treatment, providing new insights into tumour initiation and the progression of FIGC. Screening for these genotypes combined with information on the familial background may help us to identify individuals who are at increased risk of FIGC.

Materials and methods

Patients. The present study was approved by the Ethics Committee of the Third Affiliated Hospital of Nanjing University of Chinese Medicine. All study procedures were performed according to the Declaration of Helsinki ethical principles. Informed consent was obtained from all participating patients.

The proband was a 37 year-old man diagnosed with severe atrophic gastritis in 2014 by gastroscope inspection (Fig. 1). The proband's mother suffered from chronic atrophic gastritis and died of FIGC at the age of 63 years in 2014 in our hospital. His maternal grandfather and grandmother died of GC and lung cancer, respectively. The two elder brothers of his mother were dead from lung cancer and oesophagus cancer. The two younger brothers of his mother presented with chronic atrophic gastritis. His father had no family history of digestive tract diseases and was considered the normal control. The relations between individuals are illustrated in the family pedigree (Fig. 1).

Sample collection and whole-exome sequencing. A total of 5 ml of peripheral whole blood was collected from the proband and the family members listed in the family pedigree (Fig. 1). The blood samples were collected and stored at -20˚C before use.The genomic DNA was extracted using a DNA extraction kit (Youcheng Biological Pharmaceutical Technology, Co. Ltd., Jiangsu, China), following the manufacturer's instructions. The library preparation, whole-exome capture and sequencing were performed at Shanghai GeneChem, Co., Ltd. (Shanghai, China). Sequencing was analysed based on the Illumina PE150 platform (Shanghai Jeayea Biotech Co., Ltd., Shanghai, China).

The mean coverage of study samples was x100. The variant calling files were created by BCFtools and SAMtools (http://samtools.sourceforge.net). All variant annotations were performed using Variant Effect Predictor (VEP) based on the Ensemble database (http://asia.ensembl.org/info/docs/tools/vep/script/index.html).

Whole-genome analysis. Genes harbouring exonic and/or splice site variations were filtered and stratified to single-nucleotide variant (SNV) and insertion-deletion (INDEL) genes in each sample. Subsequently, the variant genes specific to patient samples were selected, which had different calls from the normal genotype and less than two reads in the normal control sample.

The genome variation profiles (accession no. GSE30833) of the blood and tumour tissues of 2 GC patients were downloaded from the public Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). The SNVs and INDELs specific to the patients from our dataset and those from the previous dataset specified above were combined to identify the overlapped somatic variants.

Function annotation of overlapped variants. The Database for Annotation, Visualization and Integration Discovery (DAVID) software allows the functional annotation of gene sets in terms of biological process (BP), molecular function (MF), cellular component (CC) and pathway. The overrepresented Gene Ontology (GO) terms in BPs and the predominant pathways were visualized by DAVID software (http://david.abcc.ncifcrf.gov/). A P-value <0.05 was set as the cut-off value of significance.

Ingenuity pathway analysis (IPA). IPA can be used to assign the functional information and biological relevance of genes in the context of known BPs, pathways and regulatory networks (12). The canonical pathways involved with variant genes were analysed by IPA software (Ingenuity Systems, Redwood City, CA, USA). A score was calculated to identify aberrant biological functions associated with the gene list.

Protein-protein interaction network analysis. Osprey served as the biological network visual tool and provided the direct and indirect protein interaction pairs (13). The protein-protein interaction network was established by the Osprey network system version 1.2.0 (Human GRID; https://osprey.thebiogrid.org/).

PCR amplification and sequencing of ESR1, ERK and IGF2R. The primers of oestrogen receptor 1 (ESR1), MAPK3/1, mitogen-activated protein kinase 3/1 (ERK1/2) and insulin-like growth factor 2 receptor (IGF2R) genes were designed by Primer 5 and synthesized by Shanghai Sangong Pharmaceutical Co., Ltd. (Shanghai, China). PCR amplification was performed with the KAPA Taq Extra system (Shanghai Jeayea Biotech Co., Ltd.) in an ABI9700 PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The PCR conditions were 94˚C for 3 min, 35 cycles at 94˚C for 20 sec, 58˚C for 15 sec and 72˚C for 3 sec, followed by a final elongation step at 72˚C for 3 min. After amplification, the PCR products were evaluated and sequenced on an ABI 3730XL automated sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Three-dimensional protein structure prediction. The Expert Protein Analysis System (ExPASy) is a web server for proteomics and protein analysis (14). Based on sequencing the genes of interest (ESR1, ERK and IGF2R), the nucleotide (DNA) sequences were translated into protein sequences using the Translate tool of ExPASy (http://web.expasy.org/translate/). The target amino acid sequences were submitted to SWISS-MODEL (https://swissmodel.expasy.org/interactive/) to produce the final 3-dimensional (3D) protein structure.

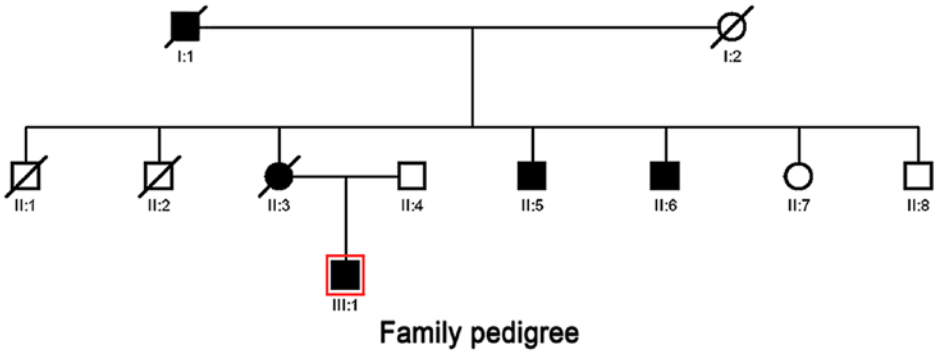

Figure 1. Family pedigree of the familial intestinal gastric cancer family members. Shaded individuals are diagnosed with gastric cancer, gastric disease or other cancers. Female and male individuals are depicted as circles and rectangles, respectively. Deceased individuals have diagonal lines through their symbol.

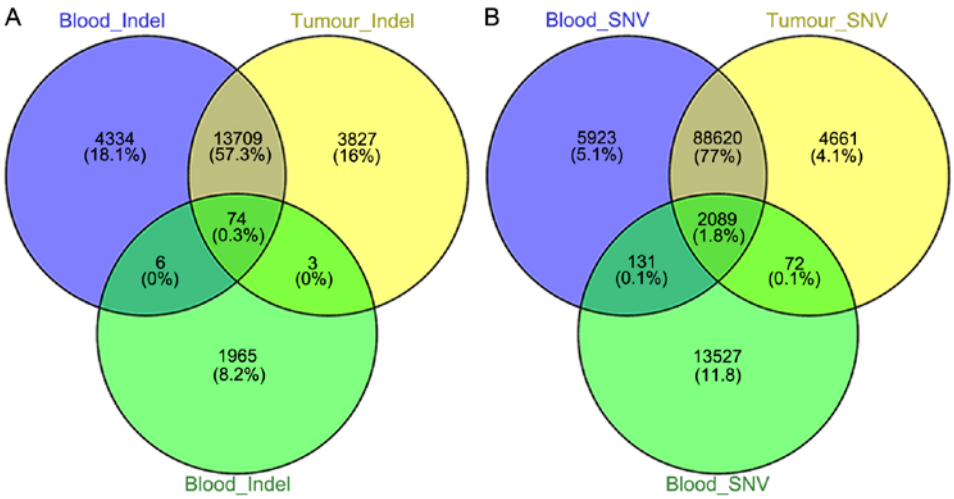

Figure 2. Venn diagram of the overlapped variant genes in the blood samples of family members with familial intestinal gastric cancer. (A) Overlapped INDELs. (B) Overlapped SNVs. Green indicates the variants from blood samples in the present study; blue and yellow indicate the variants from blood and tumour samples from the previous public study. INDELs, insertions-deletions; SNVs, single-nucleotide variants.

Immunofluorescence assay. After being embedded in paraffin, the gastric biopsy specimens of the proband were cut into consecutive 4-μm sections. The sections were incubated with the primary anti-IGF2R antibody (1:50; ab32815; Abcam, Cambridge, MA, USA) and anti-ESR1 antibody (1:40; MA5-13304; Thermo Fisher Scientific, Inc., Waltham, MA, USA) overnight at 4˚C. The sections were washed with phosphate-buffered saline (PBS) and incubated with fluorochrome-conjugated secondary antibodies, goat anti-rabbit IgG H&L (DyLight® 594) (1:200; ab96885; Abcam) and rabbit anti-mouse IgG H&L (Alexa Fluor® 488) (1:200; ab150125; Abcam), for 1 h at 37˚C. The immunofluorescence staining was observed under a fluorescence microscope (Olympus Corp., Tokyo, Japan).

Results

Data summary of the exome sequencing. In total, 571.94 M of raw reads were generated from the exome sequencing. Following quality control, 565.38 M of effective reads remained. In each sample, there were >92% of bases with a Q-value ≥30 and >96.5% of bases with a Q-value ≥20. Finally, we obtained 2048 INDELs and 15819 SNVs by exome sequencing.

Overlapped SNVs and INDELs. A Venn diagram is a simple and effective procedure that displays the overlapped gene list from different groups (15). The overlapped variant genes, compared with the public exome sequencing data of the blood and tissue samples of GC patients, are displayed in Fig. 2. The overlapped INDELs and SNVs were identified to be 74 and 2089, respectively, for further analysis.

Significant GO terms and pathways. To understand the function of gene variants at the molecular level, the SNVs and INDELs were subjected to GO and pathway enrichment analysis, respectively. As displayed in Table I, INDELs were closely associated with DNA packaging, neurological system processes and BPs related to nucleosome assembly. The significant pathways for INDELs included regulation of the actin cytoskeleton, systemic lupus erythematosus and natural-killer-cell-mediated cytotoxicity. The cell-function-related BPs were perturbed by SNVs, such as cell adhesion, motility and motion. The pathways related to cancers such as bladder, non-small cell lung, thyroid and endometrial cancer were significantly enriched by SNVs (Table I).

IPA network analysis. To identify the potential molecular function and pathways perturbed by gene variations, SNVs and

Table I. Top 10 significant GO and pathway terms associated with INDELs and SNVs.

Gene variants	Term	Count	P-value
Indels	GO:0006323~DNA packaging	4	0.008699692
	GO:0050877~neurological system process	10	0.027926677
	GO:0006334~nucleosome assembly	3	0.037114373
	GO:0031497~chromatin assembly	3	0.039558685
	GO:0065004~protein-DNA complex assembly	3	0.042910555
	GO:0050890~cognition	8	0.044035891
	GO:0034728~nucleosome organization	3	0.044625241
	GO:0016567~protein ubiquitination	3	0.06905842
	GO:0007600~sensory perception	7	0.070872214
	GO:0043087~regulation of GTPase activity	3	0.073137761
KEGG	hsa04810: Regulation of actin cytoskeleton	4	0.038043588
	hsa05322: Systemic lupus erythematosus	2	0.298474207
	hsa04650: Natural killer cell mediated cytotoxicity	2	0.379898987
SNVs	GO:0007155~cell adhesion	79	3.99E-06
	GO:0022610~biological adhesion	79	4.23E-06
	GO:0048870~cell motility	42	1.47E-05
	GO:0051674~localization of cell	42	1.47E-05
	GO:0000902~cell morphogenesis	44	1.03E-04
	GO:0006928~cell motion	54	1.40E-04
	GO:0032989~cellular component morphogenesis	47	1.61E-04
	GO:0000904~cell morphogenesis involved in differentiation	32	3.86E-04
	GO:0030855~epithelial cell differentiation	21	7.22E-04
KEGG	hsa04320: Dorso-ventral axis formation	7	0.004403386
	hsa05219: Bladder cancer	9	0.00487317
	hsa04810: Regulation of actin cytoskeleton	25	0.005877286
	hsa05223: Non-small cell lung cancer	10	0.0072363
	hsa04370: VEGF signalling pathway	12	0.008400522
	hsa04360: Axon guidance	17	0.008753884
	hsa05216: Thyroid cancer	7	0.009484706
	hsa05211: Renal cell carcinoma	11	0.014021769
	hsa04960: Aldosterone-regulated sodium reabsorption	8	0.015080678
	hsa05213: Endometrial cancer	9	0.017688902

GO, Gene Ontology; INDELs, insertions-deletions; SNVs, single-nucleotide variants; KEGG, Kyoto Encyclopedia of Genes and Genomes.

INDELs identified in our study, respectively, were subjected to IPA. As displayed in Fig. 3A the ERK1/2 (MAPK1/3) pathway, interacting with EZR and IGF2R (M6P), was the key node in the INDEL network. The major molecules such as ERK1/2, EZR and IGF2R were mainly involved in cell-to-cell signalling and closely related to interaction and connective tissue disorders and developmental disorders. As displayed in Fig. 3B, a network centred on ESR1 was constructed for SNVs. ESR1 was mainly associated with auditory disease, hereditary disorders and neurological disease, with a highest score of 44.

Protein-protein interaction. The protein interaction network with the major proteins was constructed by Osprey software. In the present study, 4 proteins (ESR1, ERK, EZK and IGF2R) were selected as the origin nodes. CDH1 was also included in particular to analyse the interactions with the four proteins related to GC. As displayed in Fig. 3C, all proteins were assembled in one protein interaction network and had direct or indirect interactions with other proteins. IGF2R demonstrated regulatory interactions with MAPK3 and MAPK1 by interacting with cellular suppressor of E1A-stimulated genes (CREG1), retinoblastoma 1 (RB1) and myelocytomatosis oncogene (MYC). ESR1 directly interacted with MAPK1, and CDH1 demonstrated a direct interaction with EZR.

Mutations of IGF2R, EZR and ESR1. The mutations of IGF2R, EZR and ESR1 were determined by PCR amplification and sequencing. The ESR1 gene showed homozygous mutations in exon 1 (216G > C) and exon 10 (2234C > T) in the proband patient. An heterozygous deletion of 68-69 GT nucleotides was detected in exon 13 of the EZR1 gene. In addition, there was an heterozygous insertion of the 1100GGGCG GGTACAGCGCGGAGGAGGAGGGAGGCC1131 nucleotide

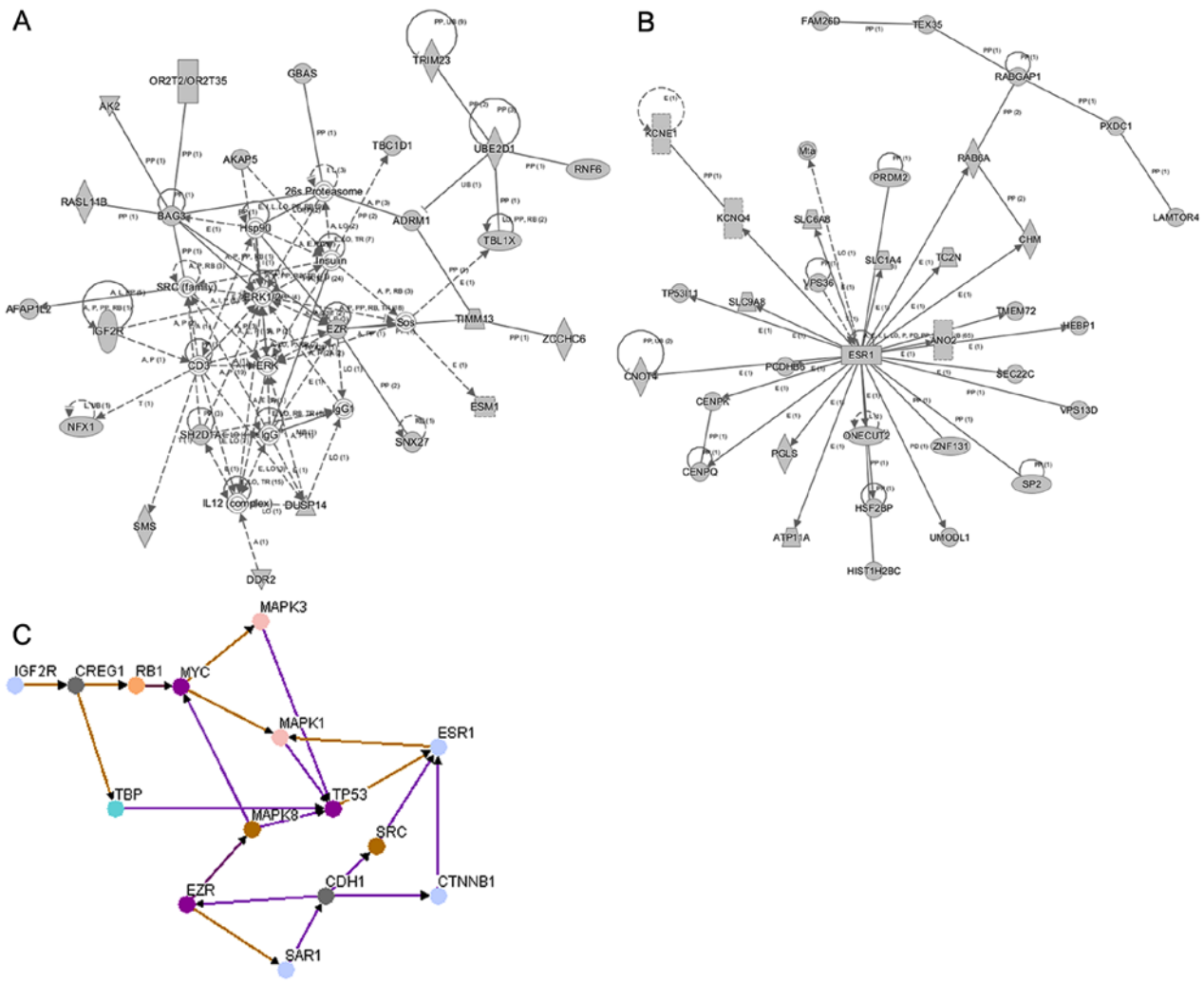

Figure 3. (A) INDEL network as analysed by IPA. (B) SNV network as analysed by IPA. (C) Protein-protein interaction network. Nodes represent hub proteins as analysed by IPA and their interacting proteins. INDEL, insertions-deletion; IPA, ingenuity pathway analysis; SNV, single-nucleotide variant.

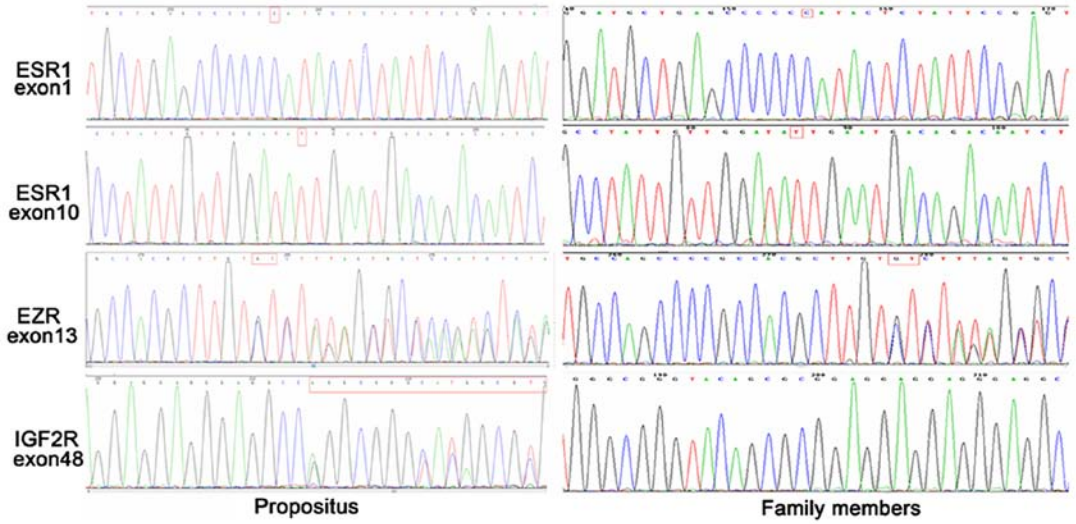

Figure 4. Sequencing diagram of the mutations in the ESR1, EZR and IGF2R genes of the propositus and his family members.

sequence in exon 48 of the IGF2R gene. ESR1 and EZR carried the same mutations in other family members, while no consistent mutations were detected for IGF2R in other family members (Fig. 4).

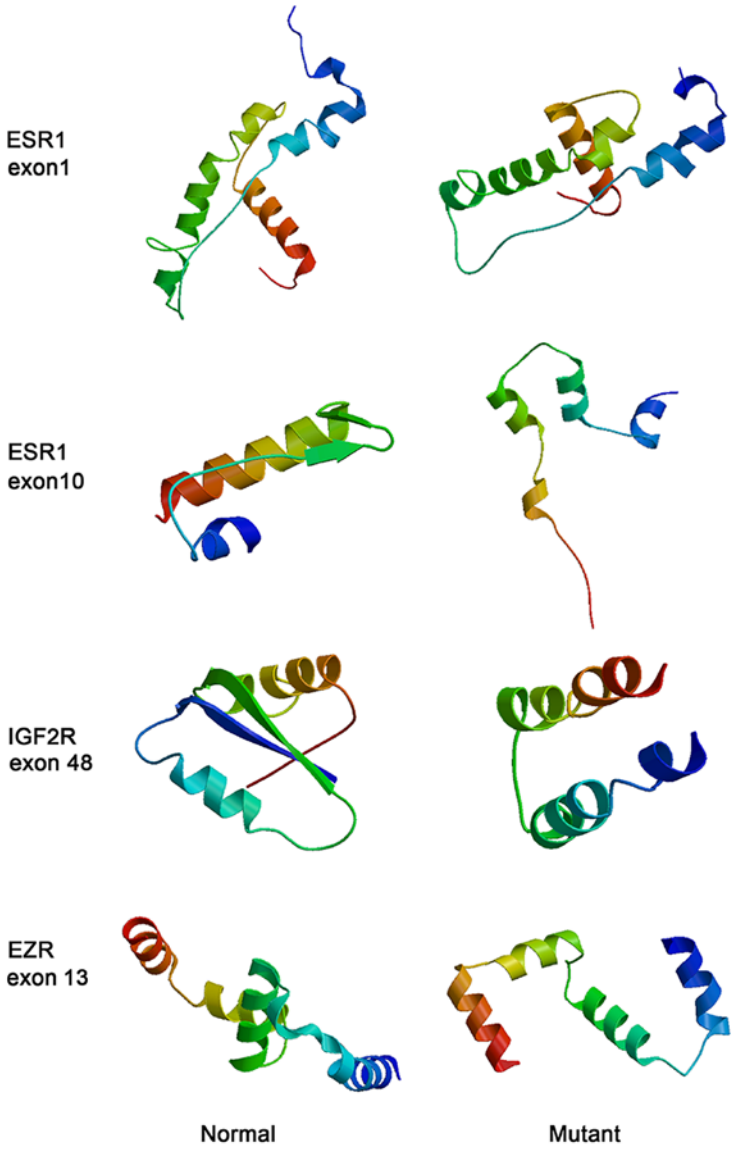

Figure 5. Prediction of the protein structure before and after mutations by SWISS-MODEL.

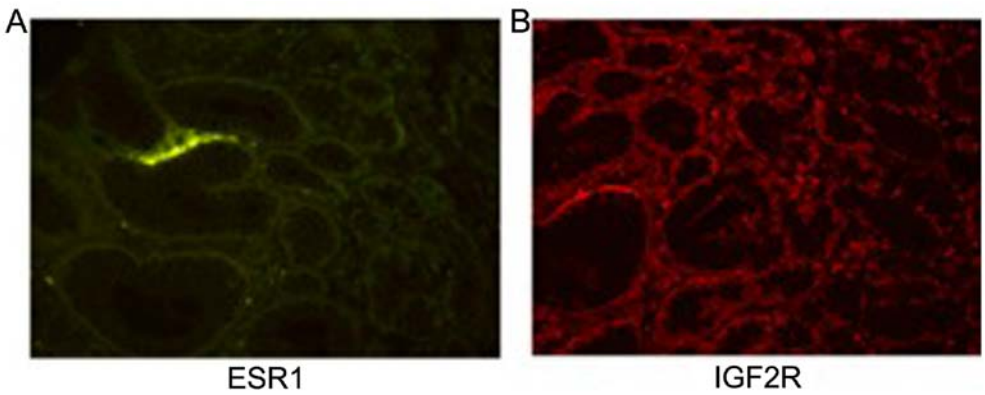

Figure 6. Immunofluorescence staining of (A) ESR1 and (B) IGF2R (magnification, x200).

Protein structure modelling. The protein structures before and after mutations were predicted by SWISS-MODEL. As illustrated in Fig. 5 the protein structures of ESR1, IGF2R and EZR became loose after mutation, particularly for ESR1 with a mutation in exon 10.

Immunofluorescence staining of IGF2R and ESR1. The immunofluorescence staining of ESR1 and IGF2R was observed under the green and red channel, respectively. As displayed in Fig. 6 the green staining was less pronounced and not specific for ESR1. Bright red staining was observed throughout the tissue section for IGF2R.

Discussion

According to Lauren's widely used histological classification, gastric cancer (GC) can be divided into intestinal and diffuse types of adenocarcinoma. Histopathologically, the Lauren GC intestinal histotype is more strongly associated with the GC familial history than the diffuse histotype (16). Incomplete intestinal metaplasia strongly increases the risk of GC and is regarded as a precursor of GC (17). Although the role of intestinal metaplasia in GC has been determined in previous studies (18) and several studies associate some genetic factors with GC among individuals with a family history of cancer (19), to date, the genetic cause for FIGC has not been well-identified. The identification of new markers predicting FIGC is important and has become the focus of intense research. Exome sequencing applied in the present study has contributed to shaping the complexity of cancers. The present study was designed to identify the putative predisposing gene defects underlying FIGC by comparing the mutation patterns with controls in a family with intestinal-type GC. In the present study, we reported an intestinal-type GC pedigree, displaying the features of family members harbouring GC with an intestinal histotype.

Based on the current dataset of exome sequencing, 2048 INDELs and 15819 SNVs were identified in the blood samples of subjects with a strong familial history of intestinal-type GC. Compared with the public exome sequencing data of the blood and tissue samples of GC patients, 74 and 2,089 overlapped genes harbouring INDELs and SNVs, respectively, were analysed. According to the GO functional enrichment analysis, the genes with INDELs were closely related to DNA packaging and nucleosome assembly, while SNV genes were enriched in cell-function-related BPs. Genes harbouring INDELs and SNVs may play different roles in the development and progression of FIGC. Pathway analysis revealed that genes harbouring SNVs were closely involved in cancer-related pathways, which proved that our findings were reliable and that genes harbouring SNVs may be centred to tumourigenesis from the intestinal metaplasia.

IPA network analysis revealed several hub genes of centrality. ERK1/2 had a remarkable centrality in the INDELs network, interacting with EZR and IGF2R. The ESR1-centred interaction network was constructed for SNVs. ERK1/2, belonging to the MAPK family, is expressed in mammalian cells (20). MAPKs, a family of mitogen-activated protein kinases, play regulatory roles in cell growth, differentiation and apoptosis (21). Accumulating evidence indicate that the activation of the MAPK/ERK signalling pathway is a common event in tumour development and invasion (22,23). The ESR1 gene, encoding oestrogen receptor α, is a well-known proto-oncogene. The activation of ESR1 induced ERK phosphorylation in a mouse spermatocyte-derived cell line, leading to apoptosis (24). In addition, IGF2R is a multiple ligand-binding cell surface receptor, the sequence of which corresponds to the bovine calcium-independent M6-P receptor. Insulin-like growth factor 2 regulates cell proliferation, apoptosis, migration and invasive ability and functions as a tumour suppressor. It has been reported that IGF2R also plays a key role in activating the downstream ERK/MAPK pathway (25). Ezrin-mediated early metastasis was reported in osteosarcoma and was partially dependent on the activation of the ERK/MAPK pathway (26). In the present study, the predicted protein-protein interaction network indicated that EZR played a regulatory role in the MAPK signalling pathway. Furthermore, previous evidence indicated that aberrant regulation of the MAPK pathway was closely associated with the development of cancer (27). Thus, we speculated that genes harbouring INDELs and SNVs perturbed the MAPK/ERK signalling pathway mediated by IGF2R and EZR, which further affected the downstream genes involved in cell apoptosis, differentiation and proliferation underlying FIGC development.

In the present study, the mutations of ESR1, EZR and IGF2R were identified in a family with intestinal-type GC by sequencing analysis. Most ESR1 mutations such as p.Leu536Gln, p.Tyr537Ser, p.Asp538Gly and D538G mutations (28-30) were identified in the ligand-binding domain (LBD) of oestrogen receptors. These mutations appear to be driver mutations, leading to a constitutively active form of ER that becomes oestrogen-independent (29). ESR1 genetic variations promote the development and progression of various cancers by altering oestrogen metabolism and play an important role in hormone binding, DNA binding and the activation of transcription to stimulate the alteration of the expression of downstream genes (30). Hypermethylation of ESR1 is associated with a loss of expression of oestrogen receptor-α, which may play a critical role in the carcinogenesis, development and prognosis of GC (31). IGF2R opposes the growth-promoting effects of IGF-2 and acts as a tumour suppressor gene for several cancers (10). It has also been demonstrated to be mutated in multiple human cancers (32,33). IGF2R was identified to be mutated in exon 27, 28 and 40 for hepatocellular carcinomas (34,35) and in exon 31 and 48 for breast cancer (36). When IGF2R is mutated, it loses its anti-oncogenic activity and neoplastic transformation may be caused by IGF 2 overaccumulation (33). IGF2R also inhibits the IGFR signalling pathway. The IGFR signalling pathway plays an important role in regulating cell proliferation, differentiation, apoptosis and development (37). As a multifunctional protein receptor, IGF2R can bind IGF2 at the cell surface and regulates the IGFR signalling pathway (10). Ezrin has been reported to have a crucial role in the dissemination of several tumours (38). However, EZR mutations in cancers have been rarely reported, particularly for GC.

To our knowledge, in the present study we reported for the first time a novel EZR deletion mutation in exon 13, ESR1 gene homozygous mutations in exon 1 (216G > C) and exon 10 (2234C > T) and an IGF2R insertion mutation in exon 48 of the intestinal-type GC family.

Protein models built by SWISS-MODEL revealed that the protein structure for ESR1, IGF2R and EZR had significant changes upon mutations. The genetic mutations may cause alterations in protein structure and affect the protein function, further altering the phenotype. Based on our findings, we speculated that the protein structure changes for ESR1, IGF2R and EZR may affect signal transduction upstream and downstream of the MAPK/ERK signalling pathway, which is related to tumourigenesis. We also speculated that the mutation of ESR1 affected protein structure and expression, leading to

the dysregulation of oestrogen signalling pathways, which may contribute to tumourigenesis. Further research is warranted to prove this hypothesis.

Currently, it is recognized that patients with a familial history of GC and precancerous conditions and lesions of the stomach may benefit from periodic surveillance (39). Therefore, for individuals with genetic mutations, we recommend intensive endoscopic surveillance annually to ensure that there is no evidence of clinically significant lesions.

Several limitations of the present study should be mentioned. Firstly, the small sample size and number of specimens limited the validation of our results. Secondly, the protein functions affected by gene mutations were not deeply investigated. Our exploratory study seeking an association between genetic variation and subjects with a family history of GC indicated that ESR1, EZR and IGF2R are candidate mutations associated with FIGC. Further biological and clinical studies should be performed to ascertain the intricate mechanisms and clinical physiological relevance of correlation in terms of mutation biology, gastric tumourigenesis and therapeutic response.

In conclusion, exome sequencing of the members of a FIGC family outlined the pathogenesis and revealed that the driving mutations in ESR1, EZR and IGF2R play the hub roles in GC pathogenesis. These mutations show potential as candidate biomarkers and as therapeutic targets in the treatment of FIGC. The variants presented here have not been previously reported. The present study provided a novel insight into the pathogenesis of GC as well as guides for the counselling of predisposed individuals.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

HC and JW conceived and designed the study. YZ and HW performed the experiments. JW wrote the paper. HC and JW reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Research Ethics Committee of The Third Affiliated Hospital of Nanjing University of Chinese Medicine and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all patients or their relatives for the use of their tissues in the experimental procedures.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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