Low IRBIT Levels Are Associated With Chemo-resistance in Gastric Cancer Patients

NOBUHIRO NAKAZAWA¹, KYOICHI OGATA¹, TAKEHIKO YOKOBORI², MUNENORI IDE³,
SEDED BAATAR¹, YASUNARI UBUKATA¹, AKIHARU KIMURA¹, NORIMICHI KOGURE¹,
MAKOTO SOHDA¹, HIROYUKI KUWANO¹, HIROSHI SAEKI¹ and KEN SHIRABE¹

¹Department of General Surgical Science, Gunma University Graduate School of Medicine, Maebashi, Japan;

²Department of Innovative Cancer Immunotherapy, Gunma University Graduate School of Medicine, Maebashi, Japan;

³Department of Diagnostic Pathology, Gunma University Graduate School of Medicine, Maebashi, Japan

Abstract. *Background/Aim:* We investigated whether the expression of inositol 1, 4, 5-trisphosphate receptor-binding protein released with inositol 1, 4, 5-trisphosphate (IRBIT) in clinical gastric cancer (GC) patients could predict the therapeutic response to postoperative adjuvant chemotherapy. *Materials and Methods:* Immunohistochemistry was used to investigate IRBIT expression in 115 GC patients. To clarify whether IRBIT had a relationship with the therapeutic effects of chemotherapy, we compared two groups – 62 patients treated with postoperative adjuvant chemotherapy and 53 patients treated with postoperative adjuvant chemotherapy. *Results:* Regarding the postoperative adjuvant chemotherapy-free group, we did not find any statistically significant correlation between clinicopathological features and recurrence regardless of the expression of IRBIT. In contrast, in the group receiving postoperative adjuvant chemotherapy, a significant association was found between IRBIT expression and both overall and disease-free survival. *Conclusion:* IRBIT may be used as a useful predictive marker for chemotherapy.

Gastric cancer (GC) is one of the most common malignancies and the third-leading cause of malignancy-related death globally (1). However, the clinical outcome of GC patients has improved their disease-free status following curative resection and postoperative adjuvant chemotherapy (2). Postoperative adjuvant chemotherapy is able to improve the survival rate after surgery; on the other hand, it remains insufficient (3). Therefore, it is important to identify predictors of the therapeutic effect of chemotherapy.

IRBIT (inositol 1, 4, 5-trisphosphate receptor-binding protein released with inositol 1, 4, 5-trisphosphate) was discovered as an inositol 1, 4, 5-trisphosphate receptor (IP3R)-binding protein (4). IRBIT is a multifunctional protein that regulates IP3R activation (5, 6), intracellular Ca^{2+} concentration (7), the BCL2L10 antiapoptotic protein (8), ion channels, and ion transporters such as the $\text{Na}^+/\text{HCO}_3^-$ co-transporter (9), the Na^+/H^+ exchanger (10), and the $\text{Cl}^-/\text{HCO}_3^-$ exchanger (11). The function of IRBIT is to suppress IP3R activation, leading to reduced IP3-induced Ca^{2+} levels (5, 6). The intracellular Ca^{2+} concentration regulates many processes as a second messenger, including cell cycle and apoptosis (7). IRBIT promotes apoptosis by inhibiting BCL2L10 and promoting contact between endoplasmic reticulum and mitochondria (8), suggesting that IRBIT is a regulator of cell death. In terms of cancer, Jeong *et al.* showed that IRBIT functions as a tumor suppressor in human epithelial ovarian cancer (12). Wittig *et al.* have shown that IRBIT is related to drug resistance against DNA-damaging drugs in a human malignant melanoma cell line (13). However, few studies have addressed the relationship between IRBIT and the therapeutic effects of chemotherapy in GC.

S-1 is an orally active combination of tegafur, gimeracil, and oteracil in a molar ratio of 1:0.4:1 (14). The results of a large-scale trial that was called the Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer (ACTS-GC) revealed that the five-year overall survival rate was 71.7% in the adjuvant S-1 group and 61.1% in the surgery-only group (15, 16). S-1 was approved

Correspondence to: Kyoichi Ogata, MD, Ph.D., Department of General Surgical Science, Graduate School of Medicine, Gunma University, 3-39-22 Showa-machi, Maebashi 371-8511, Japan. Tel: +81272208224, Fax: +81272208230, e-mail: kogata@gunma-u.ac.jp; Takehiko Yokobori, MD, Ph.D., Department of Innovative Cancer Immunotherapy, Graduate School of Medicine, Gunma University, 3-39-22 Showa-machi, Maebashi 371-8511, Japan. Tel: +81 272208222, Fax: +81 272020212, e-mail: bori45@gunma-u.ac.jp; Nobuhiro Nakazawa, MD, Department of General Surgical Science, Graduate School of Medicine, Gunma University, 3-39-22 Showa-machi, Maebashi 371-8511, Japan. Tel: +81 272208224, Fax: +81 272208230, e-mail: nakazawa75@yahoo.co.jp

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as an effective adjuvant chemotherapy in patients with stage II or III GC who underwent potentially curative surgery.

The purpose of this study was to clarify the significance of IRBIT in clinical GC patients, particularly those treated with postoperative adjuvant chemotherapy. Therefore, we performed an immunohistochemical analysis to evaluate the expression of IRBIT in GC tissues.

Materials and Methods

Patients. A total of 115 patients who underwent potentially curative surgery at the Department of General Surgical Science, Gunma University Hospital, between 2000 and 2006 were prospectively collected. To clarify whether IRBIT has a relationship with the therapeutic effects of chemotherapy, two GC groups were compared – a group of 62 patients who had not been treated with postoperative adjuvant chemotherapy and a group of 53 patients who had been treated with postoperative adjuvant chemotherapy. The characteristics of the patients in the two GC groups with and without postoperative adjuvant chemotherapy are shown in Table I. The reasons for not administering postoperative adjuvant chemotherapy were advanced age, a performance status (PS) on the Eastern Cooperative Oncology Group (ECOG) of three or four, comorbid conditions, and the inability to obtain consent. Median age, gender, lymphatic metastasis, and TNM stage differed significantly between the two GC groups with and without postoperative adjuvant chemotherapy. During the study period, the following regimens were administered as postoperative adjuvant chemotherapy: thirty nine cases were administered S-1, four cases received 5-FU, three cases each received UFT (tegafur-uracil) and FAP (5-FU plus Adriamycin plus CDDP), two cases received FP (5-FU plus CDDP), and one case each received SP (S-1 plus CDDP) and S-1 plus Paclitaxel. The postoperative pathological diagnosis of all patients was stage II or III. The GC patients were staged according to the Japanese Classification of Gastric Carcinoma: 3rd English edition, as developed by the Japanese Gastric Cancer Association (17). This study was approved by the institutional review board of Gunma University (approval no. 180246).

Immunohistochemistry. All specimens were cut into 4 μ m thick sections and mounted on glass slides. All sections were deparaffinized in xylene, rehydrated, and incubated for 30 min at room temperature in 0.3% hydrogen peroxide to block endogenous peroxidase activity. After rehydration through a graded series of ethanol treatments, antigen retrieval was carried out in Immunosaver (Nishin EM, Tokyo, Japan) at 98-100°C for 45 min. Nonspecific binding sites were blocked by incubation with Protein Block Serum-Free (DAKO, Burlingame, CA, USA) for 30 min. Samples were incubated with primary antibody (diluted by DAKO REAL antibody diluent) overnight at 4°C. The antibody for IRBIT (Proteintech Group Inc., Chicago, IL, USA, Anti-AHCYL1 antibody, 1:200 dilution) was used. Histofine Simple Stain MAX-PO (Multi) Kit (Nichirei, Tokyo, Japan) was used as the secondary antibody. Chromogen 3,3'-diaminobenzidine tetrahydrochloride was applied as a 0.02% solution in 50 mM ammonium acetate-citrate acid buffer (pH 6.0) containing 0.005% hydrogen peroxide. The sections were lightly counterstained with hematoxylin and mounted.

The tissue sections were evaluated by two independent evaluators who were blinded to the patient data. We focused on the expression

Table I. Characteristics of 115 GC patients divided into two groups, those who received (+) and those who did not receive (-) postoperative adjuvant chemotherapy.

	Adjuvant chemotherapy		p-Value
	(-) n=62	(+) n=53	
Age (years)	69	60	<0.0001*
Gender			
Male	47	30	0.029*
Female	15	23	
Histology			
Well, Moderate	25	18	0.48
Poor, Signet	37	35	
Tumor size (mm)	69	69	0.97
Depth			
m, sm, mp	9	7	0.84
ss, se, si	53	46	
Lymphatic metastasis			
Absent	16	6	0.045*
Present	46	47	
Lymphatic invasion			
Absent	6	1	0.065
Present	56	52	
Venous invasion			
Absent	46	38	0.76
Present	16	15	
Stage			
II	34	18	0.024*
III	28	35	
HER2			
Negative	55	46	0.57
Positive	5	6	
IRBIT			
Low expression	16	10	0.37
High expression	46	43	
Regimens			
S-1	–	39	–
5-FU	–	4	
UFT	–	3	
5-FU+Adr+CDDP	–	3	
5-FU+CDDP	–	2	
S-1+PTX	–	1	
S-1+CDDP	–	1	

Well: Well differentiated; Moderate: moderately differentiated; Poor: poorly differentiated; Signet: signet ring cell; Adr: Adriamycin; CDDP: cisplatin; PTX: paclitaxel. * p <0.05.

of IRBIT in cancer cells and evaluated them using the Allred score (18). The proportion of stained cells was divided into six categories (0: completely negative; 1: <1% positive; 2: 1-10% positive; 3: 11-33% positive; 4: 34-66% positive; and 5: 67-100% positive). The intensity of the most predominant area was divided into three categories (0: no staining; 1: weak positive staining; 2: moderate positive staining; 3: strong positive staining). The proportion and intensity scores were added, and the cutoff level was ≥ 5 as a high expression group in this study.

Figure 1. Representative pictures of immunohistochemical staining of IRBIT with GC tissues per field at 200 $\times$. (a) The expression of IRBIT was negative in cancerous tissue. (b) The expression of IRBIT was positive in cancerous tissue.

Figure 2. Kaplan–Meier analysis of five-year (a) overall and (b) disease-free survival in relation to IRBIT expression in patients who did not receive postoperative adjuvant therapy; in contrast, (c) overall and (d) disease-free survival in relation to IRBIT expression in patients who received postoperative chemotherapy.

Statistical analysis. Statistically significant differences were analyzed using a Mann–Whitney *U*-test for continuous variables and a chi-squared test for categorical variables. Survival rates were calculated using the Kaplan–Meier method, and statistical significance was determined using the log rank test. Univariate survival analyses were performed using the Cox proportional hazards model. A probability value of less than 0.05 was considered

to be significant. All statistical analyses were performed using JMP Pro 12.0 software (SAS Institute Inc., Cary, NC, USA).

Results

Expression of IRBIT in clinical GC tissues. Immunohistochemistry was used to investigate the expression of IRBIT

Table II. The relationship of clinicopathological factors and IRBIT expression in both groups of GC patients, those who were postoperatively treated with adjuvant chemotherapy and those who were not.

Factors	Adjuvant chemotherapy (–)			Adjuvant chemotherapy (+)		
	IRBIT					
	Low expression n=16	High expression n=46	<i>p</i> -Value	Low expression n=10	High expression n=43	<i>p</i> -Value
Age (years)	68	69	0.76	65	58	0.07
Gender						
Male	12	35	0.93	5	25	0.64
Female	4	11		5	18	
Histology						
Well, Moderate	5	20	0.39	3	15	0.77
Poor, Signet	11	26		7	28	
Tumor size (mm)	76	66	0.37	61	71	0.45
Depth						
m, sm, mp	4	5	0.19	1	6	0.73
ss, se, si	12	41		9	37	
Lymphatic metastasis						
Absent	4	12	0.93	1	5	0.88
Present	12	34		9	38	
Lymphatic invasion						
Absent	1	5	0.57	0	1	0.52
Present	15	41		10	42	
Venous invasion						
Absent	13	33	0.44	7	31	0.90
Present	3	13		3	12	
Stage						
II	9	25	0.90	3	15	0.77
III	7	21		7	28	
HER2						
Negative	14	41	0.78	9	37	0.86
Positive	1	4		1	5	

Well: Well differentiated; Moderate: moderately differentiated; Poor: poorly differentiated; Signet: signet ring cell.

in 115 GC specimens. Representative immunohistochemical staining pictures are shown in Figure 1. IRBIT was detected in the cytoplasm. Among 115 GC patients, 26 (22.6%) were classified as having low-IRBIT expression (Figure 1a), and 89 (77.4%) were assigned to the high-IRBIT-expression group (Figure 1b).

Relationship between IRBIT expression and clinicopathological features of GC patients. The relationships between the expression of IRBIT and clinicopathological features in 115 GC patients are shown in Table II. Regarding the postoperative adjuvant chemotherapy-free group, no statistical significance was found with clinicopathological features. There were also no significant associations between IRBIT expression and both overall survival and disease-free survival, as shown by the Kaplan–Meier analysis (Figure 2a and b). In contrast, regarding the group receiving postoperative adjuvant chemotherapy, a significant association was found between IRBIT expression and both overall survival ($p=0.0072$) and disease-free survival

($p=0.016$) by the Kaplan–Meier analysis (Figure 2c and d). However, the association between IRBIT expression and clinicopathological features was not statistically significant. On multivariate analyses, low expression of IRBIT in GC tissues was an independent prognostic factor of poor survival (relative Risk=3.37, 95% CI=1.41-7.56, $p=0.0076$), as was vascular invasion with postoperative adjuvant chemotherapy in 53 GC patients (Table III).

Discussion

In this study, we demonstrated that GC patients who were treated with postoperative adjuvant chemotherapy were associated with recurrence and poor prognosis, depending on the expression levels of IRBIT. Moreover, multivariate regression analysis demonstrated that IRBIT expression was an independent predictor of overall survival.

Previous studies have focused on the role of IRBIT in drug resistance against DNA-damaging drugs (10). Bonneau

Table III. Results of univariate and multivariate analyses of clinicopathological factors affecting the overall survival rate following surgery with postoperative adjuvant chemotherapy in 53 GC patients

Clinicopathological variables	Univariate analysis			Multivariate analysis		
	RR	95%CI	p-Value	RR	95%CI	p-Value
Age (<65 vs. ≥65)	0.92	0.42-2.05	0.92			
Sex (male vs. female)	1.23	0.58-2.69	0.60			
Histology type (well, moderately vs. poor, signet)	1.04	0.44-2.26	0.93			
Depth (m, sm, mp vs. ss, se, si)	2.22	0.66-13.8	0.22			
Lymph node metastasis (absent vs. present)	2.22	0.66-13.8	0.22			
Vascular invasion (absent vs. present)	2.95	1.36-6.23	0.0072*	3.36	1.53-7.22	0.0032*
IRBIT expression (low vs. high)	2.86	1.22-6.22	0.018*	3.37	1.41-7.56	0.0076*

RR: Relative risk; CI: confidence interval. * $p < 0.05$.

et al. have shown that IRBIT possesses a protein phosphatase-1 binding site (8). Interestingly, protein phosphatase-1 has been reported to mediate apoptosis *via* the dephosphorylation of Akt (19) and retinoblastoma protein (20, 21). These findings suggested that IRBIT is a target of protein phosphatase-1. In this study, we compared the postoperative adjuvant chemotherapy-free group with the group receiving postoperative adjuvant chemotherapy. No statistically significant association was found between clinicopathological features and recurrence. In addition, poor prognosis in the postoperative adjuvant chemotherapy-free group depended on the expression of IRBIT. However, a significant association was found between IRBIT expression and the presence of recurrence and prognosis in the group receiving postoperative adjuvant chemotherapy. Our results suggest that IRBIT is involved in chemotherapy resistance. Similarly to our results in GC, Jeong *et al.* have shown that IRBIT functions as a tumor suppressor in human epithelial ovarian cancer (9). Hence, low IRBIT may be predictive of a higher risk of GC recurrence in patients treated with postoperative adjuvant chemotherapy.

Arnaoutov and Dasso have shown that IRBIT knockdown lead to interphase length variability and accelerated mitotic progression in HeLa cells (22). This finding suggests that IRBIT controls cell-cycle progression. This study also suggests the possibility that the regulation of ribonucleotide reductase, which supplies the building blocks necessary for DNA synthesis and repair, controls genomic stability and ensures proper cell-cycle regulation by IRBIT. In this study, the most frequent postoperative chemotherapeutic regimens used were S-1 and 5-FU. Tegafur, one of the constituent elements of S-1, is a prodrug of 5-fluorouracil (5-FU). 5-FU acts in several ways, but mainly as a thymidylate synthase inhibitor. Interfering with the action of this enzyme prevents the synthesis of pyrimidine thymidine, which is a nucleoside required for DNA replication. Both IRBIT and 5-FU with S-1 were thought to be related to DNA replication. In light of

these findings and our results, it was considered that the expression of IRBIT could induce the effect of 5-FU with S-1. Furthermore, IRBIT would be critical for developing personalized treatments for patients based on patient risk assessments.

Our study has several limitations. First, this study has a small sample size, which may bias the results of our study. Further large-scale clinical trials are needed to clarify the potential of IRBIT as a new predictive biomarker for postoperative chemotherapy. Second, we compared patients who did not receive postoperative adjuvant chemotherapy with those who did. Recently, S-1 plus oxaliplatin and capecitabine plus oxaliplatin as well as S-1 have been approved as postoperative adjuvant chemotherapy for GC patients. Thus, other regimens of postoperative adjuvant chemotherapy also need to be evaluated.

In conclusion, we clarified that the low expression of IRBIT in GC tissues obtained from patients who had been treated with postoperative adjuvant chemotherapy was associated with recurrence and poor prognosis. IRBIT could be useful as a predictive marker for chemotherapy.

Conflicts of Interest

The Authors declare that they have no conflict of interest regarding this study.

Authors' Contributions

Conception and design: N. Nakazawa, K. Ogata, and T. Yokobori; Acquisition of data: N. Nakazawa, S. Baatar, Y. Ubukata, A. Kimura, and N. Kogure; Analysis and interpretation of data: N. Nakazawa, K. Ogata, T. Yokobori, and K. Shirabe; Writing, review, and/or revision of the manuscript: N. Nakazawa, K. Ogata, T. Yokobori, M. Sohda, H. Kuwano, H. Saeki, and K. Shirabe; Material support: M. Ide; Study supervision: K. Ogata, T. Yokobori, M. Sohda, H. Kuwano, H. Saeki, and K. Shirabe. All Authors have read and approved the final manuscript.

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