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BRIEF ARTICLE

Loss of BRCA1 expression leads to worse survival in patients with gastric carcinoma

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Abstract

AIM: To investigate the expression deficiency of key molecular markers in the homologous recombination pathway.

METHODS: Expression loss of breast cancer type 1 susceptibility protein (BRCA1), ataxia telangiectasia mutated (ATM), ATM-Rad3-related (ATR), mediator of DNA damage checkpoint protein 1 (MDC1) and meiotic recombination 11 (Mre11) were correlated with their clinicopathological parameters in gastric cancer (GC). One hundred and twenty treatment-naive GC samples were formalin-fixed and paraffin-embedded into tissue blocks. Two representative cores from each block were extracted and constructed into tissue microarrays. Expression levels of BRCA1, ATM, ATR, MDC1 and Mre11 were determined using immunohistochemical analysis, and correlated with clinical parameters, including age, gender, Lauren subtype, tumor grades, clinical stage and overall survival.

RESULTS: Expression loss of BRCA1, ATM, ATR, MDC1, and Mre11 was found in 21.4%, 20.2%, 21.0%, 11.1% and 4.6%, respectively, of interpretable cases. BRCA1 loss was significantly associated with patients of diffused subtype (intestinal *vs* diffused, 8.2% *vs* 31.7%, $P = 0.001$), higher tumor grade (Ⅰ/Ⅱ *vs* Ⅲ, 10.7% *vs* 20.5; Ⅰ/Ⅱ *vs* Ⅳ, 10.7% *vs* 54.5%, $P = 0.047$) and advanced clinical stage (Ⅰ/Ⅱ *vs* Ⅲ, 12.9% *vs* 16.9%; Ⅰ/Ⅱ *vs* Ⅳ, 12.9% *vs* 45.5%, $P = 0.006$). MDC1 loss was significantly associated with patients of diffused subtype (intestinal *vs* diffused, 0% *vs* 19.7%, $P = 0.001$) and higher tumor grade (Ⅰ/Ⅱ *vs* Ⅲ, 0% *vs* 12%; Ⅰ/Ⅱ *vs* Ⅳ, 0% *vs* 30.8%, $P = 0.012$). In addition, the survival time of the patients with expression loss of BRCA1 was significantly shorter than those with positive expression of BRCA1 (2-year survival rate, 32.4% *vs* 62.8%, $P = 0.015$). No correlations were found between clinicopathological parameters and expression loss of ATM, ATR and Mre11.

CONCLUSION: Our results support the hypothesis that homologous recombination deficiency plays an important role in the progression of gastric carcinoma. Loss of expression of BRCA1 and MDC1 may serve as predictive factors in tumor development or progression in GC patients.

Key words: Homologous recombination deficiency; Gastric cancer; Breast cancer type 1 susceptibility protein; Mediator of DNA damage checkpoint protein 1; Ataxia telangiectasia mutated; Ataxia telangiectasia mutated-Rad3-related

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INTRODUCTION

DNA lesions constantly threaten the integrity of our genome. Of the major DNA lesions, double-strand DNA breaks (DSBs) pose the most dangerous threat[1]. DSBs occur when both complimentary strands of DNA break simultaneously, and failure to repair these DSBs can result in chromosomal aberrations including mutations, deletions, amplifications, translocations, all of which can lead to cancer predispositions. Cells employ two major pathways to repair DSBs: homologous recombination (HR) and non-homologous end joining (NHEJ). HR and NHEJ differ mainly in two aspects. First, they differ in the frequency of errors that occur during DSB repairs. NHEJ employs a direct ligation mechanism that is highly error-prone, while HR utilizes the genomic information stored in homologous strands to proof-read the repair process and thus is essentially error-free. Second, the two pathways differ in the cell cycles in which they are primarily involved. NHEJ is most commonly found in G0 and G1 phases; meanwhile HR predominates in S and G2 phases, which are two critical stages that require high-fidelity transmission of genetic information. Attributed to its error-free mechanism and deployment in key cell-cycle phases, HR plays a central role in the protection against DSBs and hence is crucial in maintaining the genomic stability of the cells[2].

A complex and hierarchical network of proteins is implicated in the HR pathway to detect, signal and repair DSBs. In this network, breast cancer type 1 susceptibility protein (BRCA1), ataxia telangiectasia mutated (ATM), ATM-Rad3-related (ATR), mediator of DNA damage checkpoint protein 1 (MDC1) and meiotic recombination 11 (Mre11) are most important functionally. In brief, ATM/ATR located at the top of the signaling cascades act as the core sensors of DSBs[3] by collaborating with other sensor molecules, including MDC1[4] and the complex of MRE11-Rad50-NBSI[5]. Downstream substrates that are involved in checkpoint activation, among them BRCA1/2[6], are then phosphorylated by ATM and ATR, causing cell cycle arrest until DSBs are repaired[7].

Defects in the HR pathway or homologous recombination deficiency (HRD) directly compromise the genomic stability and predispose to cancer formation[8]. The relationship between HRD and development of many cancer types has been well established[9]. Genetic aberrations of BRCA1/2, the most widely studied markers in the HR pathway, have been found to promote both tumor initiation and progression[10,11]. These genetic abnormalities, together with BRCA1/2 protein loss, were reported in many carcinomas[12], especially in breast cancer (BC) and ovarian cancer (OC). In BC, *BRCA1/BRCA2* mutations are responsible for 3%-8% of all cases and 30%-40% of familial cases. Ten percent of patients with OC have a genetic predisposition. About 80% of families with a history of OC have *BRCA1* mutations, while 15% have *BRCA2* mutations[13]. Aberrations in ATM function are linked with head and neck squamous cell carcinoma[14], chronic lymphocytic leukemia[15], colorectal cancer[16], BC and OC[17]. Genetic alterations of ATR were frequently reported in BC and OC[18-20]. Dysfunctional MDC1 was implicated in BC development[21,22] among other cancer types[23,24]. Abnormal Mre11 signaling is strongly linked with BC, with mutations and protein loss found to be associated with BC pathogenesis[25-28].

These HRD tumors also demonstrated enhanced sensitivity toward DNA-damaging agents, through the so-called "synthetic lethality"[29]. These specific populations of tumor cells, under DNA-damaging agents, such as poly (ADP-ribose) polymerase (PARP) inhibitors[30], are unable to recruit the necessary cellular machinery for the repair of DSBs and will undergo apoptosis. Pre-clinical studies of PARP inhibitors had raised the expectations for this highly selective therapeutic approach in HRD patients although these hypotheses need to be further validated in the clinical studies[31,32]. Therefore, it is useful to understand the status of HRD-specific markers in different tumor types, such as GC.

GC is the second leading cause of cancer-related death worldwide and is particularly prevalent in Asia. Previous reports suggested that HRD could play a role in the carcinogenesis of the stomach[33-37]. Yet, HRD's prognostic perspective in GC has not been fully explored and this study aims to address these questions. In order to assess the involvement of HRD in gastric tumorigenesis, we have analyzed the immunohistochemical expression of BRCA1, ATM, ATR, MDC1 and Mre11 in 120 GC samples and correlated them with clinicopathological parameters.

MATERIALS AND METHODS

Clinical samples and patient information

One hundred and twenty formalin-fixed and paraffin-embedded (FFPE) tissue samples were collected from Shanghai Renji Hospital for the study. All patients underwent radical resection between 2007 and 2010. The median age of the patients (82 males and 38 females) was 61.3 years (range: 22-87 years). All tumor tissues were diagnosed with gastric adenocarcinomas by two qualified pathologists.

Immunohistochemistry

GC tumor tissue and adjacent non-tumor tissue samples were collected after surgery following standard FFPE procedure. Tissue microarray (TMA) was then made with 2 representative cores withdrawn from FFPE block for each case. Four μm-thick tissue sections were cut from TMA for immunohistochemical (IHC) study. The slides were baked at 56 ℃ for 1 h, then de-paraffinized in xylene for 20 min and rehydrated through a graded series of ethanol concentrations (5 min in 100% ethanol first, followed by 5 min in 70% ethanol). Antigen retrieval was done in pressure cooker for 5 min using Target Retrieval Solution (Dako, Copenhagen, Denmark). Endogenous peroxidase activity was blocked by Peroxidase Blocking Reagent (Dako, Copenhagen, Denmark) for 5 min. Primary antibodies (ATM, 1:50, Epitomics, cat. No. 1549-1; ATR,

Table 1 Association between expression loss of homologous recombination markers and clinicopathological parameters in gastric cancer patients *n* (%)

	BRCA1 expression (*n* = 112)		ATM expression (*n* = 114)		ATR expression (*n* = 86)		MDC1 expression (*n* = 117)		Mre11 expression (*n* = 86)	
	BRCA1-negative/ total cases	*P* value	ATM-negative/ total cases	*P* value	ATR-negative/ total cases	*P* value	MDC1-negative/ total cases	*P* value	Mre11-negative/ total cases	*P* value
Age, yr (median)										
< 61.3	15 (25.0)	0.043	9 (16.1)	0.133	8 (19.5)	0.193	8 (14.3)	0.625	2 (4.9)	0.119
⩾ 61.3	9 (17.3)		14 (24.1)		10 (22.2)		5 (8.2)		2 (4.4)	
Gender										
Male	15 (19.7)	0.284	15 (19.5)	0.715	12 (21.1)	0.969	10 (12.7)	0.430	3 (5.0)	0.333
Female	9 (25.0)		8 (21.6)		6 (20.7)		3 (7.9)		1 (3.4)	
Lauren type										
Intestinal	4 (8.2)	0.001	12 (24.0)	0.846	8 (21.6)	0.891	0 (0.0)	0.001	2 (5.0)	0.303
Diffused	20 (31.7)		11 (17.2)		10 (20.4)		13 (19.7)		2 (4.3)	
Tumor grade										
Ⅰ / Ⅱ	3 (10.7)	0.047	7 (24.1)	0.513	5 (29.4)	0.327	0 (0.0)	0.012	1 (4.2)	0.742
Ⅲ	15 (20.5)		15 (20.5)		10 (16.7)		9 (12.0)		3 (5.8)	
Ⅳ	6 (54.5)		1 (8.3)		3 (33.3)		4 (30.8)		0 (0.0)	
Clinical stage										
Ⅰ / Ⅱ	4 (12.9)	0.006	6 (19.4)	0.560	6 (23.1)	0.593	3 (9.4)	0.092	2 (7.7)	0.562
Ⅲ	10 (16.9)		11 (17.7)		7 (16.7)		5 (7.7)		2 (4.2)	
Ⅳ	10 (45.5)		6 (28.6)		5 (27.8)		5 (25.0)		0 (0.0)	

BRCA1: Breast cancer type 1 susceptibility protein; ATM: Ataxia telangiectasia mutated; ATR: ATM-Rad3-related; MDC1: Mediator of DNA damage checkpoint protein 1; Mre11: Meiotic recombination 11.

1:100, Santa-Cruz Technology, cat. No. sc-1887; BRCA1, 1:100, Merck, cat. No. OP92; MDC1, 1:500, Sigma, cat. No. M2444; Mre11, 1:200, Abcam, cat. No. ab214) were then applied to cover the specimen for 1 h at room temperature, followed by incubation with labeled polymer-HRP anti-rabbit or anti-mouse secondary antibody (Dako, Copenhagen, Denmark) for 30 min at room temperature. Thorough rinsing with TBST was done after incubation with each reagent. The slides were visualized using DAB substrate-chromagen (Dako, Copenhagen, Denmark) and washed with deionized water before counterstaining with haematoxylin. The slides were then dehydrated through a graded series of ethanol concentrations, cleared in xylene and coverslipped in DPX mounting medium.

Immunohistochemical scoring

The intensity of the staining in the nuclear of tumor cells was recorded. Scoring was established as follows: 0, if absence of staining was observed; 1+, if the tumor cells had weak staining; 2+, if tumor cells had moderate staining; and 3+, if tumor cells had strong staining. Tumors with 1+, 2+ and 3+ expression were interpreted as positive and tumors with no expression (0 score) were interpreted as expression loss. Given the heterogeneity of protein expression in tumor cells, the highest scoring from either one of TMA cores was counted as the final result.

Statistical analysis

The analysis was conducted with SPSS 16.0 software. Characteristics of the two groups were compared using the χ^2 likelihood ratio test. Logistic regression model was applied to interrogate association of IHC data and individual clinical parameter. The Kaplan-Meier method was used to estimate the survival distributions. The log-rank test was used to compare the survival distributions. Two-sided *P* values < 0.05 were considered statistically significant.

RESULTS

Among the 120 cases, 69.2% of tumors (83/120) involved the ventricular sinuses, 14.1% (17/120) involved the ventricle corpora and 16.7% (20/120) involved the cardia in the stomach. All tumor samples were diagnosed with adenocarcinoma with different tumor grade and Lauren subtypes.

The overall follow-up rate is 87% with a median follow-up time of 32 mo. At the time of analysis, 49.2% (49/120) patients were alive and 50.8% (61/120) patients died. The overall 2-year survival rate was 54.2%. Loss of BRCA1 expression was observed in 21.4% (24/112), ATM in 20.2% (23/114), ATR in 20.9% (18/86), MDC1 in 11.1% (13/117), and Mre11 in 4.7% (4/86) of the GC patients (Figure 1). Clinicopathological parameters and expression of HRD biomarkers in the samples are displayed in Table 1.

Expression loss of each marker and its correlation with clinicopatholofical parameters

The clinicopathological parameters of patients in the study included age, gender, Lauren type, tumor grade and clinical stage according to 2010 World Health Organization tumor-node-metastesis classification. Statistical analysis of IHC data and clinicopathological parameters are shown in Table 1. Loss of ATM, ATR and Mre11 expression was not associated with gender or clinical stage. BRCA1 loss was significantly associated with patients of diffused subtype ($P = 0.001$), higher tumor grade ($P = 0.047$) and advanced clinical stage ($P = 0.006$). MDC1

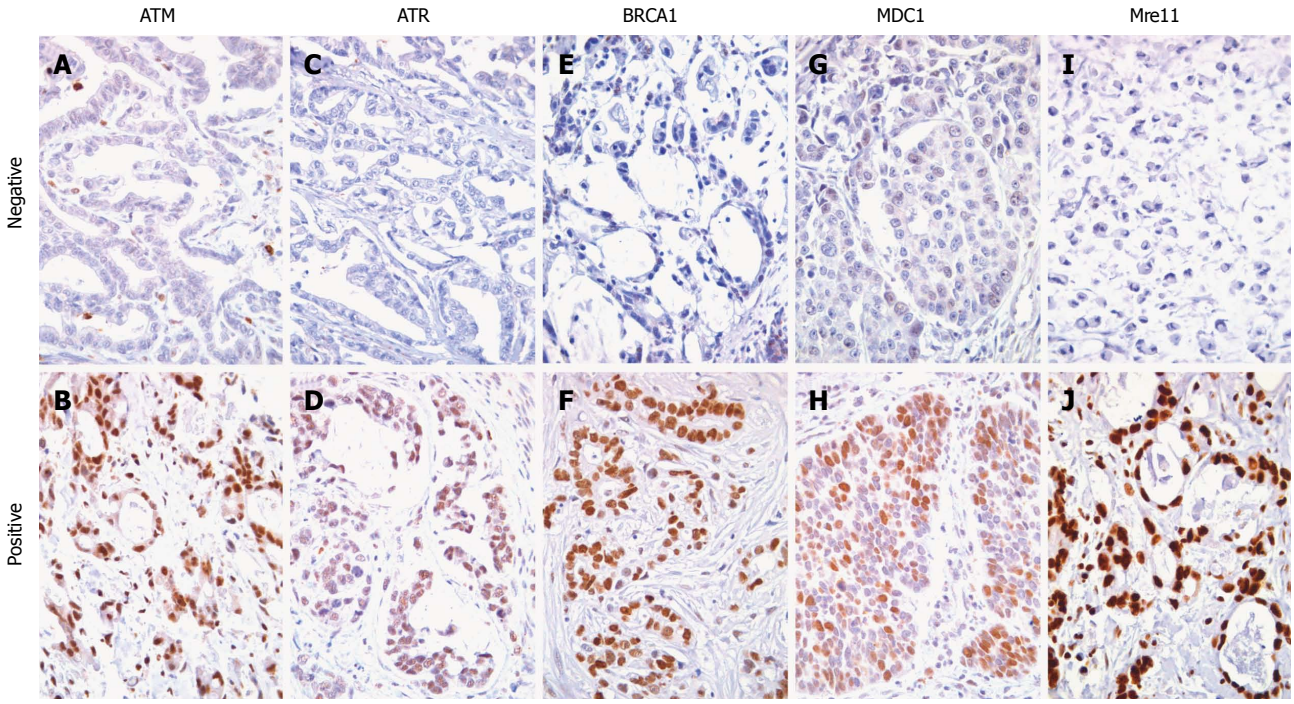

Figure 1 Immunohistochemical expression of ataxia telangiectasia mutated, ataxia telangiectasia mutated-Rad3-related, breast cancer type 1 susceptibility protein, mediator of DNA damage checkpoint protein 1 and meiotic recombination 11 in gastric cancer tissues. ATM: Ataxia telangiectasia mutated; ATR: Ataxia telangiectasia mutated-Rad3-related; BRCA1: Breast cancer type 1 susceptibility protein 1; MDC1: Mediator of DNA damage checkpoint protein 1; Mre11: Meiotic recombination 11. 3,3'-Diaminobenzidine staining, ×200.

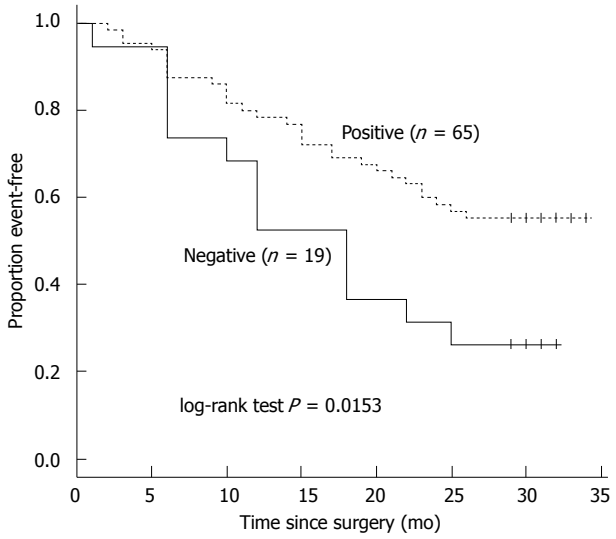

Figure 2 Negative effect of breast cancer type 1 susceptibility protein loss on patient overall survival. The survival time of the patients with positive expression of breast cancer type 1 susceptibility protein (BRCA1) was significantly longer than those with negative expression of BRCA1.

loss was significantly associated with patients of diffused subtype ($P = 0.001$) and higher tumor grade ($P = 0.012$).

Correlation between BRCA1 expression and survival

Expression loss of BRCA1 was significantly associated with the progression of the GC patients. The survival time of the patients with BRCA1 expression loss was significantly shorter than BRCA1-positive patients (2-year survival rate, 32.4% *vs* 62.8%, $P = 0.015$; Figure 2). Expression of the other four markers was not significantly associated with survival ($P > 0.05$).

Combined biomarker analysis

Twenty-seven (51.9%, 27/52) cases had positive expression of all 5 protein kinases (HR+ group) and 25 (48.1%, 25/52) cases had expression loss of at least one protein kinase (HRD group). Significant difference of tumor grade was observed between the two groups, with the HRD group showing significant association with higher tumor grades ($P = 0.013$). But there was no significant difference in gender, Lauren type or clinical stage. Survival analysis also showed no significant difference between the two groups.

DISCUSSION

Gastric cancer is one of the leading causes of cancer-related death worldwide, and although the incidence has decreased in Western countries, Asia remains the specific high-risk area. Various reports have suggested that HRD could play a role in gastric tumorigenesis. However, a systematic analysis of the key markers in the HR pathway is largely missing. In the present study, the expression losses of the five key markers, namely BRCA1, ATM, ATR, MDC1 and Mre11 were correlated with the clinicopathological parameters in a cohort of Chinese GC patients.

Recent studies of the relationship between BRCA1

and tumors mainly focused on BC and OC. The frequency of BRCA1 mutations among breast cancer patients is less than 5%[38], while the loss of BRCA1 protein expression is higher at around 20%[39]. However, BRCA1 expression in gastric cancer has rarely been studied. Our data showed that BRCA1 expression deficiency occurred in 24/112 (21.4%) GC patients. BRCA1 deficiency was significantly associated with patients of diffused Lauren type, higher tumor grades and advanced clinical stage. Patients with BRCA1 deficiency lived significantly shorter ($P = 0.015$) than those patients with positive expression of BRCA1, indicating that loss of BRCA1 can serve as a prognostic marker. Mutations of *BRCA1* in gastric cancer were not found commonly[40]. Rather, microsatellite instability and loss-of-heterozygosity of *BRCA1* gene at locus D17S855 were shown to be the predominant genetic abnormalities found in GC[41]. Both of these genetic instabilities may lead to the reduction or loss of the functional BRCA1 protein. Recently, a high frequency of hypermethylation on the BRCA1 promoter was found in tumor tissues and these epigenetic changes correlated with the loss or reduction of protein expression[42]. These reports together with our data, suggest that BRCA1 protein loss may be a suitable indicator of cancer development in GC.

Lack of reports on *MDC1* mutations suggests that down-regulation of the marker at the protein level may serve as a better prognostic marker. MDC1 protein loss/reduction was previously described[22], although its correlation with survival was not assessed. Patel *et al*[21] addressed this question by profiling MDC1 in subsets of early-stage BC patients who underwent breast-conserving surgery and radiation therapy and found that decreased MDC1 was not related to overall survival. However, they found that MDC1 reduction correlated with nodal failure and concluded the role of MDC1 in early cancer development. To our knowledge, our study is the first to assess MDC1 expression in GCs. The strong association between MDC1 deficiency and diffused subtype indicates that MDC1 plays a major role in this subtype's development. In addition, the association between MDC1 and higher tumor grade also suggests that MDC1 deficiency is implicated in GC pathogenesis. Although MDC1 loss failed to establish a significant correlation with survival, the strong linkage of MDC1 loss with diffused type and higher tumor grades warrants further research into this marker.

Our data suggested ATM, ATR and Mre11 deficiencies were commonly found in GC patients. But there was no significant difference in clinicopathological features between the patients with negative and positive expression for each marker. Mutations of *ATM* have been suggested to play a possible role in the carcinogenesis of other cancer types. The rate of *ATM* mutations in advanced GC has been previously studied and although several variants were found, there were no hot spots. In the same study, decreased level of phosphorylated ATM at Ser1981 significantly correlated with poor differentiation, lymph node metastasis and poor 5-year survival[43]. Mutation of *ATR* was previously reported in BC[19], OC[18] and colon cancers[44], but has never been found in GC. In addition, protein loss of ATR has never been studied in GC and we report here for the first time that protein loss of ATR is a common feature in GC. We investigated whether Mre11 mutation could play a role in GC. In a previous study[45] that correlated MRE11 poly(T)11 mutations with clinicopathological features, a significant association was found only in patients with a family history of GC. In addition, the authors demonstrated that this *MRE11* mutation was associated with absent or strongly reduced Mre11 immunostaining, indicating that protein loss of Mre11 may be a suitable surrogate for the detection of Mre11-related HRD in GC. In our study, the same antibody (Clone 12D7) for the detection of Mre11 was used and the results agreed with those from the previous studies.

In the combined biomarker analysis, we found significant difference in tumor grade between the HR+ and HRD groups, under the assumption that loss of one protein kinase is sufficient to cause a non-functional HR pathway. Our data suggested that HR deficiency played an important role in the GC pathogenesis but is not necessarily crucial in gastric tumor maintenance. Further work will be done to address whether significant association would appear when a larger patient population and a longer follow-up time are available.

These results have made possible the clinical use of DNA-damaging agents in HRD GCs, although finding markers that could predict response is still a daunting challenge[46]. While most of the PARP inhibitors in BC and OC employed *BRCA1/2*-mutation as the patient selection criteria[47,48], this may not be the best strategy in GC, as protein loss is evidently the driver. For the other HRD biomarkers, their prognostic and predictive values need to be further investigated. In our opinion, unless they are validated in both pre-clinical and clinical settings, BRCA1 remains the strongest predictor of response to compounds that are exploiting the HRD pathway.

COMMENTS

Background

DNA lesions constantly threaten the integrity of our genome. Of the major DNA lesions, double-strand DNA breaks (DSBs) pose the most dangerous threat. DSBs occur when both complimentary strands of DNA break simultaneously, and failure to repair these DSBs can result in chromosomal aberrations, including mutations, deletions, amplifications and translocations, all of which can lead to cancer predispositions.

Research frontiers

Various reports have suggested that homologous recombination deficiency (HRD) could play a role in gastric tumorigenesis. However, a systematic analysis of the key markers in the homologous recombination pathway is largely missing. In the present study, the expression losses of the five key markers, namely breast cancer type 1 susceptibility protein (BRCA1), ataxia telangiectasia mutated, ataxia telangiectasia mutated-Rad3-related, mediator of DNA damage checkpoint protein 1 and meiotic recombination 11, were correlated with the clinicopathological parameters in a cohort of Chinese gastric carcinoma (GC) patients.

Innovations and breakthroughs

The results have made possible the clinical use of DNA-damaging agents in HRD GCs, although finding markers that could predict response is still a daunting challenge.

Applications

For the other HRD biomarkers, their prognostic and predictive values need to be further investigated. In author's opinion, unless they are validated in both pre-clinical and clinical settings, BRCA1 remains the strongest predictor of response to compounds that are exploiting the HRD pathway.

Peer review

This is an interesting study in which authors investigated the expression deficiency of key molecular markers in the HR pathway. The results are interesting and suggest that homologous recombination deficiency plays an important role in the progression of GC.

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