Published in final edited form as:

Tumour Biol. 2016 June ; 37(6): 8057–8066. doi:10.1007/s13277-015-4682-6.

A variant at a potentially functional microRNA-binding site in *BRIP1* was associated with risk of squamous cell carcinoma of the head and neck

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Abstract

DNA double-strand breaks (DSBs) are one of the most serious forms of DNA damage to the cell, causing genomic instability and ultimately carcinogenesis. In this study, we hypothesized that single nucleotide polymorphisms (SNPs) at the micro RNA (miRNA)-binding sites of DSB repair genes may influence cancer risk by dysregulating target gene expression. To test our hypothesis, we firstly performed functional prediction for common SNPs in DSB genes and found 12 potentially functional SNPs located at the miRNA-binding sites. We then investigated their associations with risk of squamous cell carcinoma of the head and neck (SCCHN) in 1087 patients and 1090 cancer-free controls in a non-Hispanic white population. As a result, SNP rs7213430 in *BRIP1* was found to be significantly associated with cancer risk (P_{trend} = 0.021). Compared with the AA homozygotes, the G allele carriers had an increased risk of SCCHN (adjusted OR 1.16, 95 % CI 1.02–1.31). Marginal significance was found for another SNP rs15869 in *BRCA2* (*P* = 0.053). Further, functional analyses showed that SNP rs7213430 is within the *miR-101* seed-

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Electronic supplementary material The online version of this article (doi:10.1007/s13277-015-4682-6) contains supplementary material, which is available to authorized users.

Compliance with ethical standards

Conflicts of interest

None

binding region, and the variant G allele could lead to significantly lower luciferase activity and *BRIP1* mRNA expression, compared to the A allele with the presence of *miR-101*. Our results suggested that SNP rs7213430 in the 3′-UTR of *BRIP1* might contribute to SCCHN susceptibility by affecting the binding activity of *miR-101* and resulting in a decreased *BRIP1* expression. Additional larger population and functional studies are warranted to confirm our findings.

Keywords

DNA double strands break; MicroRNA; Genetic susceptibility; Head and neck cancer; Polymorphism

Introduction

Squamous cell carcinoma of the head and neck (SCCHN), a group of cancers involving the oral cavity, pharynx, and larynx, is one of the six most common cancers worldwide [1]. In the USA, approximately 60,000 new cases are expected to be diagnosed with 12,000 deaths in 2015 [2]. Etiologic studies have identified multiple risk factors for SCCHN [3–6], of which tobacco smoke and alcohol use are the well-known risk factors for most types of SCCHN. Infection of human papillomavirus (HPV), especially HPV-16, also plays a major role in the development of oropharyngeal cancer. In addition to those known environmental factors, recent studies have suggested that genetic factors (e.g., genetic variants) also contribute to the disease [7–9].

DNA damage response is known to protect against carcinogenesis in vivo, and a deficient response could lead to carcinogenesis through mechanisms of altered expression of genes such as those regulated by miRNAs [10–12]. Micro RNAs (miRNAs) are shown to play essential roles in the DNA damage and repair pathway, and overall proficient miRNA expression levels should be maintained to elicit a proper DNA damage and repair response as a barrier against cancer development [12]. SNPs located in the miRNA-binding sites (e.g., 3′-UTR) may affect regulation and function of miRNA-mediated genes and are thus associated with individual susceptibility to cancer development, including SCCHN [13–15]. However, the role of genetic variants in miRNA-binding sites of DNA double-strand break (DSB) repair pathway genes in SCCHN susceptibility is largely unknown.

In the present study, to test the hypothesis that genetic variants in the predicted miRNA-binding sites of DSB repair genes are associated with risk of SCCHN, we firstly performed bioinformatics predictions for SNPs in the selected DNA DSB repair pathway genes with a minor allele frequency (MAF) of ≥0.05 in European populations and identified 12 SNPs located at the 3′-UTR of five DSB repair genes (*BRCA2*, *BRIP1*, *NBS1*, *RAD51*, and *XRCC3*) with potential functions to influence the binding activity of miRNAs. We further investigated the influence of those 12 SNPs on cancer risk in 1087 non-Hispanic white SCCHN cases and 1090 cancer-free controls frequency-matched on age, sex, and ethnicity. For those identified SNPs, we also evaluated their functions on gene expression by the luciferase assay and real-time reverse-transcription polymerase chain reaction (RT-PCR) assay in cancer cell lines and peripheral blood mononuclear cells (PBMCs).

Materials and methods

Study population

The subjects' characteristic details of this hospital-based case-control study had been previously reported elsewhere [14]. Briefly, the study population included 1087 non-Hispanic white patients with newly diagnosed, untreated primary tumors of the oral cavity (n = 319, 29.3 %), oropharynx (n = 553, 50.9 %), and larynx or hypopharynx (n = 215, 19.8 %) seen at The University of Texas M.D. Anderson Cancer Center during the period between October 1999 and October 2007. By using the frequency matching on age (±5 years), sex, and ethnicity, we also identified an additional 1090 cancer-free controls from among hospital visitors at The M.D. Anderson Cancer Center during the same time period. Patients with second SCCHN primary tumors, primary tumors of the nasopharynx or sinonasal tract, or any histopathologic diagnosis other than SCCHN were excluded. Having given a written informed consent, each eligible subject provided additional information about risk factors, such as tobacco smoking and alcohol use, as well as a one-time sample of 30 ml of blood for biomarker tests. Among 1090 cancer-free controls, 105 subjects who had leftover frozen PBMCs with different genotypes for the selected SNPs were used for evaluating messenger RNA (mRNA) expression levels. The University of Texas M.D. Anderson Cancer Center Institutional Review Board approved the research protocol.

Selection and genotyping of the miRNA binding sites SNPs

The methods for the bioinformatics prediction of putative miRNA-binding sites had been described previously [16]. Briefly, the miRNA target prediction was carried out by using online tools available at http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm [17]; http://mrsnp.osu.edu/ [18, 19]; http://cmbi.bjmu.edu.cn/mirsnp [20] and http://www.targetscan.org/ [21]. We also searched the National Institute of Environmental Health Sciences Genome Program's SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP) and related literature to identify all potentially functional SNPs in the DNA DSB repair pathway genes with a minor allele frequency ≥0.05 in European populations. As a result, 12 SNPs, which are located in the predicted miRNA-binding sites, were selected for further investigation. The effects of SNPs on the miRNA-target interaction were classified into four groups, labeled as create, break, decrease, or enhance according to previously described [20] (Supplementary Table 1).

We extracted genomic DNA from the buffy coat fraction of the whole blood samples by using a blood DNA mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The DNA purity and concentration were determined by spectrophotometer measurement of absorbance at 260 and 280 nm. The 12 miRNA-binding site SNPs in the five DNA DSB repair genes were genotyped by using the TaqMan methodology in 384-well plates, which were read with the Sequence Detection Software on an ABI-Prism 7900HT instrument according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Primers and probes were supplied by Applied Biosystems. Each plate included four negative controls (no DNA), duplicated positive controls, and eight repeat samples. Amplification was done under the following conditions: 50 °C for 2 min, 95 °C for 10 min,

and 60 °C for 1 min for 40 cycles. For all genotypes, the assay success rate was >99 %, and the repeated samples' results were 100 % concordant.

RT-PCR analysis for mRNA expression levels of *BRIP1* and *BRCA2* in PBMCs

The mRNA expression levels of *BRIP1* and *BRCA2* were examined by quantitative RT-PCR with samples of the total RNA that was isolated from PBMCs of 105 cancer-free controls by using the TRIzol reagent (Invitrogen™, Carlsbad, CA). *BRIP1* and *BRCA2* mRNA expression levels were detected by using the TaqMan gene expression assays with the master mix reagent (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Each amplification reaction was performed in a final volume of 5 μl containing 5 ng of the cDNA, 0.25-μl primers, and 2.5-μl Master mix. Real-time RT-PCR was performed using the ABI-Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The 5-μl reaction mixtures were incubated in a 384-well optical plate at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Each sample was analyzed in duplicate, and the expression levels of *BRIP1* and *BRCA2* mRNA were calculated relative to the expression level of *18S rRNA*.

Reporter constructs, transfection and luciferase assays

The 687-bp fragment of the *BRIP1* 3′-UTR containing the A or G allele at rs7213430 was amplified with the forward primer 5′-GTAGACGCGTAAAGGCATGTTTCCTGGTTTT-3′ (*Mlu*I) and the reverse primer 5′-TCCAAAGCTTGCGAAATATGACTGAGGTGTCA-3′ (*Hind*III) from a homozygous human genomic DNA sample. The PCR products were separated in agarose gel and extracted, purified, and cloned into pMIR-REPORT plasmids (Applied Biosystems) with *Mlu*I and *Hind*III digestion. The head and neck cancer cell line (UM-SCC-1, UMSCC14A and MDA686LN) were seeded 0.5×10^5 cells per well in 24-well plates (BD Biosciences, Bedford, MA), and 24 h after the plating, cells were co-transfected with the FuGENE HD reagent (Roche Applied Science, Indianapolis, IN, USA). Each co-transfection reaction contained 500 ng of pMIR-BRIP1 (rs7213430) A or G vector plus 50-ng pRL-TK plasmids (Promega, Madison, WI) that served as a transfection internal control and along with 50 pmol of miR-101-5P RNA (Sigma-Aldrich, Atlanta, GA). At 48 h after transfection, cells were washed and lysed with 100-μl Passive Lysis Buffer (Promega, Madison, WI). The luciferase activities of both firefly and renilla luciferase were quantified by a Dual-Luciferase Reporter Assay System (Promega, Madison, WI) and the relative luciferase activity was obtained, according to the manufacturer's instructions (BD Monolight™ 3010 Luminometer, Becton Dickinson Company, Mississauga, ON, Canada). Physical and biological containment procedures of recombinant DNA used in this study were practiced in accordance with the US National Institutes of Health. The experiments were performed in independent triplicate for all samples. Differences were determined by Student *t* test, and $P < 0.05$ was considered significant.

Statistical analysis

We evaluated differences in selected demographic variables, risk factors and genotype frequencies of the DSB repair genes between cases and controls by using the χ^2 test and examined Hard-Weinberg equilibrium by a goodness-of-fit χ^2 test to compare the observed

genotype frequencies with the expected ones among the controls. The associations of SNPs of DSB repair genes with risk of SCCHN were estimated by computing the odds ratios (ORs) and their 95 % confidence intervals (CIs) from both univariate and multivariate logistic regression models. These analyses were performed with or without adjustment for age (in years), sex, smoking status, and alcohol use. The stratified analysis of identified SNPs was also performed by age, sex, smoking and drinking status, tumor site, and tumor stage. Subjects who had smoked <100 cigarettes in their lifetime were defined as never smokers; all others were defined as ever smokers. Among ever smokers, those who had quit and had not smoked for >1 year were defined as former smokers, and the others were defined as current smokers. Similarly, subjects who had reported drinking alcoholic beverages at least once a week and longer than 1 year prior to diagnosis or interview were defined as ever drinkers. Those who had quit drinking for longer than 1 year prior to diagnosis or interview were defined as former drinkers and the others as current drinkers. All tests were two sided, and $P < 0.05$ was considered significant. All statistical analyses were performed with SAS software (version 9.1.3; SAS Institute, Inc., Cary, NC), unless stated otherwise.

Results

The final analysis included 1087 SCCHN patients and 1090 cancer-free controls adequately matched on age (cases versus controls [mean ± standard deviation]: 57.1 ± 11.2 years versus 56.7 ± 11.0 years, $P = 0.547$), and sex ($P = 0.525$) (Table 1). The cases were more likely than the controls to be current smokers (37.8 % versus 14.5 %) or current drinkers (50.9 % vs. 40.5 %). Furthermore, the differences in tobacco smoke and alcohol use between cases and controls were statistically significant (both $P < 0.001$). All of these variables were further adjusted for any confounding effects in later multivariate logistic regression analysis.

Table 2 summarizes the genotype distribution of each SNP and their association with risk of SCCHN. The genotype distributions of the 12 SNPs in the controls were in agreement with the Hardy-Weinberg equilibrium ($P > 0.05$), indicating that the chance of genotyping errors was small. The most notable finding was that SNP rs7213430 (A >G) in the *BRIP1* gene was significantly associated with risk of SCCHN. Compared with the wild-type genotype (AA), the variant G genotypes were associated with an increased risk of SCCHN (adjusted OR = 1.14, 95 % CI = 0.94–1.38, $P = 0.199$ for AG and adjusted OR = 1.35, 95 % CI = 1.05–1.75, $P = 0.021$ for GG), showing a significant allele-dosage effect ($P_{trend} = 0.021$). We also found a borderline significance for the association between the *BRCA2* rs15869 variant CC genotype and SCCHN risk, compared with the wild-type AA genotype (adjusted OR = 1.60 and 95 % CI = 0.99–2.57, $P = 0.053$). No significant associations were found for other SNPs examined in this study (Table 2). We then examined the combined effects of these two SNPs by calculating the number of risk genotypes (i.e., rs7213430GG and rs15869CC). As shown in Table 3, when we used “0” risk genotypes as the reference, the “1–2” risk genotype group had a significantly increased risk of SCCHN (adjusted OR = 1.33, 95 % CI = 1.07–1.65; $P = 0.010$).

In an exploratory analysis, we further stratified the risk associations of *BRIP1* rs7213430 A >G and *BRCA2* rs15869 A >C by various host characteristics. The results showed that the

increased risk associated with the variant genotypes of rs7213430 and rs15869 was more evident in the younger subjects (adjusted OR = 1.54, 95 % CI = 1.12–2.13 for rs7213430), never drinkers (adjusted OR = 2.47, 95 % CI = 1.06–5.77 for 15869). Additionally, the increased risk associated with the combined risk genotypes was also more pronounced among the younger subjects (adjusted OR = 1.57, 95 % CI = 1.16–2.12, $P = 0.036$), males (adjusted OR = 1.28, 95 % CI = 1.00–1.65, $P = 0.055$), never smokers and never drinkers (adjusted OR = 1.58 and 1.57, 95 % CI = 1.12–2.23 and 1.10–2.24, $P = 0.010$ and 0.014, respectively), oropharyngeal (adjusted OR = 1.34, 95 % CI = 1.03–1.73, $P = 0.027$), and stage III–IV (adjusted OR = 1.31, 95 % CI = 1.04–1.65, $P = 0.025$) (Table 4).

To further characterize biological significance of the *BRIP1* rs7213430 and *BRCA2* rs15869, we conducted correlation analysis between the two SNPs and corresponding mRNA expression in PBMCs samples from 105 cancer-free controls. In this subset of samples, 39 had the rs7213430 AA genotype, 53 had the AG genotype, and 13 had the GG genotype for the *BRIP1*, in agreement with the Hardy-Weinberg equilibrium ($P = 0.439$). As shown in Fig. 1a, The *BRIP1* mRNA relative expression was lower in subjects with the rs7213430 GG genotype (mean ± SD, 0.500 ± 0.048) than in those with the AA genotype (mean ± SD, 0.533 ± 0.038) and the AA/AG genotypes (mean ± SD, 0.535 ± 0.036), and the differences were statistically significant ($P = 0.016$ and 0.002, respectively); for the *BRCA2*, 73 had the rs15869 AA genotype, 30 had the AC genotype, and two had the CC genotype, which was also in agreement with the Hardy-Weinberg equilibrium ($P = 0.588$). However, as shown in Fig. 1b, the relative *BRCA2* mRNA expression levels for the rs15869 CC, AC, and AC/ CC genotypes were not significantly different from that for the AA genotype ($P = 0.430$, 0.077 and 0.125, respectively). Because the other SNPs were not associated with SCCHN risk, their correlations with the related mRNA expression were not further investigated.

It has been reported that the 3′-UTR of *BRIP1* contains the potential binding sites for miR101-5p [22] (http://mrsnp.osu.edu) (Fig. 2a). The RNA folding and hybridization prediction showed that rs7213430 A-to-G allele substitution leads to the minimal free energy (MFE) changed from −12.3 to −17.3 kcal/mol (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/sumbmission.html), indicating that the 3′-UTR sequence containing G allele has a higher binding affinity with miR101-5p than the sequence containing the A allele (Fig. 2b). We further test the influence of different alleles of rs7213430 A >G in the 3′-UTR of *BRIP1* on the activity of miRNA-101-5p by the luciferase assay. We first replaced the 3′-UTR of a luciferase reporter gene with the 687-bp *BRIP1* 3′-UTR containing either rs7213430A or rs7213430G. All constructs used in this study were verified by directing sequencing (Fig. 2c). As shown in Fig. 2d, both constructs with the A and G alleles had a relatively decreased luciferase activity, compared with the control vector. In addition, significantly lower levels of luciferase expression were observed, when co-transfected with miR101-5p and *BRIP1* 3′-UTR luciferase reporter plasmids carried the rs7213430 G allele, compared with the that carried the A allele (UM-SCC-1 cell line: 0.535 ± 0.050 for G allele versus 0.774 ± 0. 135 for the A allele, $P = 0.048$, UMSCC14A: 0.460 ± 0.037 for G allele versus 0.674 ± 0.083 for the A allele, $P = 0.015$, MDA686LN, 0.326 ± 0.052 for G allele versus 0.678 ± 0.049 for the A allele, $P < 0.001$).

Discussion

In this hospital-based case-control study, we evaluated associations between 12 common SNPs at the miRNA-binding sites of five major DSB repair genes and risk of SCCHN in a non-Hispanic white population. We found that the G allele of rs7213430 in the *BRIP1* gene was associated with a significantly increased risk of SCCHN. We also provided biological evidence that the *BRIP1* rs7213430 G allele was associated with lower mRNA expression of *BRIP1* than the A allele by influencing the binding activity of miR101-5p. These data suggest that the miRNA-binding site SNP (rs7213430) may play a role in the etiology of SCCHN by mediating the mRNA expression levels of *BRIP1*.

The BRIP1 (BRCA1-interacting protein 1), which belongs to the DEAH helicase family [23, 24], is an essential tumor suppressor gene. It has been demonstrated that *BRIP1*-encoded helicase could interact with BRCA2, playing an important role in controlling BRCA1-dependent DNA repair, DNA damage-induced G2-M checkpoint control, and possibly tumor suppression [23, 25–27]. Although the exact mechanism underlying the *BRIP1*-related tumor susceptibility remains unknown, *BRIP1* mutations have been shown to influence genomic stability and risk of multiple cancers. For example, sequence variants of *BRIP1* have been reported to be associated with an increased risk of several types of cancer, such as that of prostate, breast, and ovaries [28–30]. These studies provided evidence for the role *BRIP1* may play in genetic susceptibility to cancer. More recently, two studies investigated the association of the miRNA-binding site SNP of *BRIP1* rs7213430 with risk of breast and ovarian cancers in Chinese populations [31, 32]. In one relatively small study (306 cases and 319 controls in a Chinese population), Ren et al. found that the variant GG genotype of *BRIP1* rs7213430 was associated with significantly reduced risk of breast cancer [31]. In another case-control study of 298 cancer cases and 286 controls, the authors also found that the rs7213430 was associated with a reduced risk of cervical cancer [32]. However, no studies investigated the association between this SNP and SCCHN risk. In the present study, we found that the variant GG genotype of rs7213430 was associated with statistically significantly increased risk of SCCHN and decreased levels of gene expression, compared with the AA and AA/AG genotypes in the population study and functional study, respectively. Our results are not only consistent with those previous mutation studies but also suggest that *BRIP1* is a tumor-suppressor gene in the etiology of SCCHN. The inconsistence between our findings and the two previous population studies in Chinese populations may be due to different population structures or tumor heterogeneity across the studies of different cancer types.

Previous functional studies have revealed that the interaction between BRCA2 and BRIP1 play a key role in enhancing error-free DNA damage repair and DNA damage check-point control [20, 25]. In the present study, we observed that the combined risk genotypes of the two SNPs of *BRCA2* rs15869 and *BRIP1* rs7213430 were associated with risk of SCCHN in a risk-genotype dose-response manner; in particular, the subjects with 1 to 2 risk genotypes had a significantly increased risk of SCCHN compared with those with 0 risk genotypes. This finding implies that the SNPs of *BRCA2* and *BRIP1* genes may have a joint effect on risk of SCCHN. Furthermore, when comparing 1–2 risk genotypes with 0 risk genotype, the risk of SCCHN was higher in never smokers and never drinkers than in ever smokers and

ever drinkers, indicating that the risk in non-smokers and non-drinkers may be more likely to be genetically determined in the absence of strong exposure to smoking and alcohol use. Another finding in the present study was that the combined risk genotypes were significantly higher for oropharyngeal cancer, but not for non-oropharyngeal cancers. The difference in risk for patients at different sites may result from different etiologies for oropharyngeal (more HPV-infection oriented) and non-oropharyngeal (more smoking and alcohol use oriented) cancers. This finding may suggest that *BRCA2* and *BRIP1* genes may have interaction with oncogenic proteins of HPV, which are a major risk factor for oropharyngeal cancer. However, this hypothesis needs to be tested in future studies.

There are several limitations of this study. Firstly, although our study had over 1000 SCCHN cases and 1000 controls, the sample size may still not have an enough statistical power to identify weak effects of the SNPs investigated or interactions between SNPs and environmental factors. Secondly, in the present study, our results indicate that the *BRIP1* SNP rs7213430, located in the miR-101-binding site, is likely to disrupt miRNA-target interaction, resulting in the alteration of *BRIP1* mRNA expression, a possible underlying mechanism for the observed association with increased risk of SCCHN. However, the exact mechanism for the effect of rs7213430 on the binding activity of miRNA101 and *BRIP1* needs further functional studies. As there is still lack evidence to support the gene function of *BRIP1* on the development of SCCHN, additional functional assays and in vitro models are required to verify our findings. Lastly, due to the retrospective nature of the original case-control study design, we did not have reliable information on HPV infection that most likely had caused oropharyngeal cancer, and it is also unclear about the underlying mechanism of the *BRIP1* gene expression and HPV infection status. These issues may be addressed in future studies with larger sample size and detailed clinical information.

In summary, our results suggested that SNP rs7213430 in the 3′-UTR of *BRIP1* might contribute to SCCHN susceptibility by affecting the binding activity of *miR-101* and resulting in decreased *BRIP1* expression. Additional population or functional studies are warranted to confirm our findings.

Acknowledgments

We thank Margaret Lung and Jessica Fiske for their assistance in recruiting the subjects and gathering the questionnaire information and Qiming Wang, Peng Li, Jianzhong He, and Min Zhao for their laboratory assistance.

This study was supported by the National Institutes of Health grant R01 ES111740-11 (to Q. W.), and a start-up fund (to Q. Wei) from Duke Cancer Institute. QW was also supported from the Duke University Medical Center and the Duke Cancer Institute as part of the P30 Cancer Center Support Grant (NIH CA014236).

Abbreviations

DSB	DNA double-strand break
SNP	Single nucleotide polymorphism
SCCHN	Squamous cell carcinoma of the head and neck
PBMCs	Peripheral blood mononuclear cells

OR	Odds ratio
CI	Confidence interval

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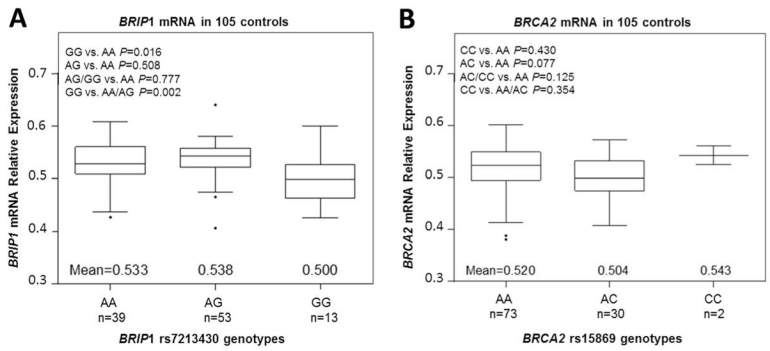

Fig. 1.

Correlation analysis of two SNPs (*BRIP1* rs7213430 and *BRCA2* rs15869) and the relative mRNA expression levels of *BRIP1 and BRCA2* in peripheral blood mononuclear cells (PBMCs) of 105 cancer-free controls. **a** The relative *BRIP1* mRNA expression level was lower in PBMCs with rs7213430 GG genotype (0.500 ± 0.048) than in those with the AA genotype (0.533 ± 0.038) and AA/AG genotypes (0.535 ± 0.036), and these differences were statistically significant ($P < 0.016$, $P = 0.002$, respectively). **b** The relative *BRCA2* mRNA expression levels were similar among the four groups with the *BRCA2* rs15869 AA, AC, CC, and AA/AC genotypes (0.520 ± 0.041 for AA, 0.504 ± 0.044 for AC, 0.543 ± 0.025 for CC, and 0.515 ± 0.042 for AA/AC genotypes). There was no any significant correlation between the genotypes and mRNA expression levels of *BRCA2*

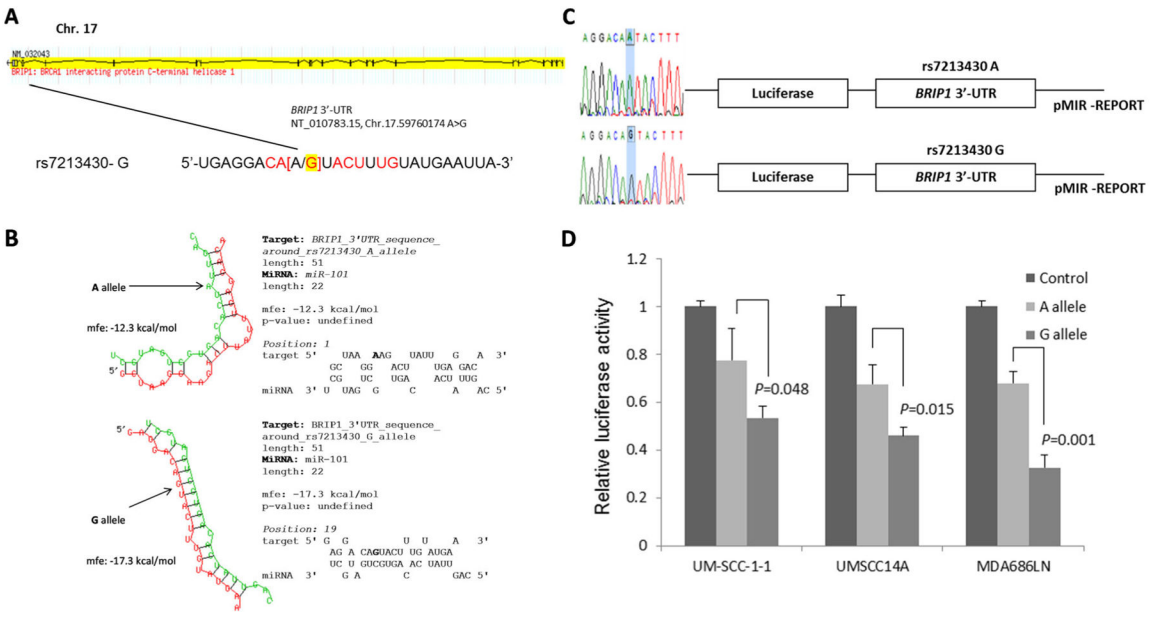

Fig. 2.

Characterization and functional analysis of the *BRIP1* 3′-UTR. **a** The sequence around SNP rs7213430 and its location within the *BRIP1* 3′-UTR. This SNP is located within the seed region of the binding site with the G allele, perfectly matching the corresponding C allele in *miR-101.* The red-labeled nucleotides are complementary to those in *miR-101.* **b** The miRNA-101 folding and hybridization prediction showed that rs7213430 A to G allele substitution led to the minimal free energy (MFE) changed from −12.3 to −17.3 kca/mol. **c** Schematic drawing of the reporter gene constructs containing a 687-bp *BRIP1* 3′-UTR region; the only difference between the two constructs was an A or G polymorphic site, and **d** Luciferase activity assays to measure the A/G allele difference at rs7213430. Head and neck cancer cell lines were transiently transfected with A- or G-containing reporters and co-transfected with miRNA 101 momic. All constructs were co-transfected with pRL-TK Renilla plasmid as internal control. Results are shown as the relative percentages of the luciferase activity. Data were from three independent transfection experiments with assays conducted in six replicates. *P* values were determined by two-sided Student's *t* test

Table 1

Frequency distributions of selected variables in SCCHN cases and cancer-free controls

Variables	Cases (*n* = 1087)		Controls (*n* = 1090)		*P*[a]
	n	Percent	*n*	Percent	
Age (years)					
≤50	299	27.5	312	28.6	0.547
51–57	281	25.9	260	23.9	
>57	507	46.6	518	47.5	
Sex					
Female	270	24.8	258	23.7	0.525
Male	817	75.2	832	76.3	
Smoking status					
Never	304	28.0	535	49.1	<0.001
Former	372	34.2	397	36.4	
Current	411	37.8	158	14.5	
Alcohol use					
Never	296	27.2	473	43.4	< 0.001
Former	238	21.9	176	16.1	
Current	553	50.9	441	40.5	
Tumor site					
Oropharynx	553	50.9			
Non-oropharynx [b]	534	49.1			

[a] Two-sided χ^2 test

[b] Included oral cavity (*n* = 319), larynx (*n* = 172), and hypopharyngeal (*n* = 43) cancer cases

Table 2

Genotype frequencies of the polymorphisms of DSB repair genes among SCCHN cases and control subjects and their associations with SCCHN risk

Genotypes	Cases, *n* (%)[a]	Controls, *n* (%)[a, b]	Adjusted *OR* (95 % CI)[c]	*P*[c]
BRCA2 rs15869				
AA	655 (60.6)	691 (63.7)	1.00 (reference)	
AC	376 (34.8)	362 (33.4)	1.06 (0.88–1.28)	0.540
CC	50 (4.6)	32 (2.9)	1.60 (0.99–2.57)	0.053
P, Trend			1.13 (0.97–1.32)	0.115
Recessive model	50 (4.6)	32 (0.8)	1.57 (0.98–2.51)	0.060
BRCA2 rs11571836				
AA	633 (58.6)	634 (58.3)	1.00 (reference)	
AG	398 (36.8)	395 (36.3)	1.00 (0.84–1.20)	0.978
GG	50 (4.6)	59 (6.4)	0.87 (0.58–1.30)	0.482
P, Trend			0.97 (0.84–1.13)	0.731
Recessive model	50 (4.6)	59 (6.4)	0.87 (0.58–1.29)	0.484
BRCA2 rs7334543				
AA	611 (56.5)	577 (53.1)	1.00 (reference)	
AG	402 (37.2)	434 (40.0)	0.92 (0.77–1.10)	0.365
GG	68 (6.3)	75 (6.9)	0.89 (0.62–1.27)	0.503
P, Trend			0.93 (0.81–1.07)	0.313
Recessive model	68 (6.3)	75 (6.9)	0.92 (0.65–1.30)	0.624
BRIP1 rs7213430				
AA	355 (32.7)	394 (36.2)	1.00 (reference)	
AG	526 (48.4)	522 (48.0)	1.14 (0.94–1.38)	0.199
GG	205 (18.9)	172 (15.8)	1.35 (1.05–1.75)	0.021
P, Trend			1.16 (1.02–1.31)	0.021
Recessive model	205 (18.9)	172 (15.8)	1.26 (1.00–1.58)	0.052
NBS1 rs1063053				
CC	497 (46.0)	520 (47.8)	1.00 (reference)	
CT	471 (43.6)	454 (41.7)	1.06 (0.88–1.28)	0.531
TT	113 (10.4)	114 (10.5)	0.98 (0.73–1.32)	0.917
P, Trend			1.02 (0.90–1.17)	0.743
Recessive model	113 (10.4)	114 (10.5)	0.96 (0.72–1.28)	0.787
NBS1 rs1063054				
TT	495 (45.6)	520 (47.7)	1.00 (reference)	
GT	476 (43.9)	452 (41.5)	1.09 (0.90–1.31)	0.380
GG	114 (10.5)	118 (10.8)	0.97 (0.72–1.30)	0.847
P, Trend			1.02 (0.90–1.17)	0.749
Recessive model	114 (10.5)	118 (10.8)	0.94 (0.71–1.24)	0.642
NBS1 rs2735383				
CC	491 (46.2)	518 (48.0)	1.00 (reference)	

Genotypes	Cases, *n* (%)[a]	Controls, *n* (%)[a, b]	Adjusted *OR* (95 % CI)[c]	*P*[c]
CG	465 (43.7)	445 (41.3)	1.06 (0.89–1.28)	0.507
GG	107 (10.1)	115 (10.7)	0.92 (0.68–1.24)	0.579
P, Trend			1.01 (0.89–1.26)	0.539
Recessive model	107 (10.1)	115 (10.7)	0.90 (0.68–1.20)	0.484
RAD51 rs11855560				
CC	358 (33.1)	349 (32.1)	1.00 (reference)	
CT	461 (42.7)	465 (42.8)	0.96 (0.78–1.17)	0.665
TT	262 (24.2)	273 (25.1)	0.93 (0.74–1.17)	0.522
P, Trend			0.97 (0.86–1.09)	0.571
Recessive model	262 (24.2)	273 (25.1)	0.96 (0.78–1.17)	0.652
RAD51 rs12593359				
GG	287 (26.4)	275 (25.2)	1.00 (reference)	
GT	533 (49.1)	541 (49.6)	0.95 (0.77–1.17)	0.632
TT	266 (24.5)	274 (25.2)	0.93 (0.73–1.19)	0.572
P, Trend			0.97 (0.86–1.09)	0.591
Recessive model	266 (24.5)	274 (25.2)	0.97 (0.79–1.18)	0.731
RAD51 rs7180135				
AA	362 (33.4)	357 (32.8)	1.00 (reference)	
AG	530 (48.9)	520 (47.9)	1.00 (0.82–1.21)	0.972
GG	192 (17.7)	210 (19.3)	0.91 (0.71–1.17)	0.473
P, Trend			0.96 (0.85–1.09)	0.542
Recessive model	192 (17.7)	210 (19.3)	0.92 (0.73–1.14)	0.435
XRCC3 rs709399				
AA	554 (51.1)	513 (47.7)	1.00 (reference)	
AG	212 (19.6)	224 (20.8)	1.08 (0.90–1.30)	0.397
GG	318 (29.3)	339 (31.5)	0.82 (0.57–1.17)	0.265
P, Trend			0.94 (0.85–1.04)	0.251
Recessive model	318 (29.3)	339 (31.5)	0.93 (0.77–1.23)	0.460
XRCC3 rs861536				
AA	424 (59.2)	451 (41.5)	1.00 (reference)	
AG	497 (45.9)	471 (43.3)	1.17 (0.98–1.41)	0.105
GG	162 (14.9)	165 (15.2)	1.03 (0.79–1.33)	0.847
P, Trend			1.05 (0.93–1.19)	0.448
Recessive model	162 (14.9)	165 (15.2)	0.95 (0.74–1.21)	0.663

[a]The numbers were not the same for each single nucleotide polymorphism because of their different calling rates due to few uncalling samples

[b]The observed genotype frequency among the control subjects were in agreement with Hardy-Weinberg equilibrium ($P = 0.541$ for *BRCA2* rs15869, $P = 0.257$ for *BRCA2* rs11571836, $P = 0.494$ for *BRCA2* rs7334543, $P = 0.539$ for *BRIP1* rs7213430, $P = 0.144$ for *NBS1* rs1063053, $P = 0.127$ for *NBS1* rs1063054, $P = 0.400$ for *NBS1* rs2735383, $P = 0.392$ for *RAD51* rs11855560, $P = 0.328$ for *RAD51* rs12593359, $P = 0.120$ for *RAD51* rs7180135, $P = 0.328$ for *XRCC3* rs709399, $P = 0.120$ for *XRCC3* rs861536)

[c]Adjusted by age, sex, smoking status, and alcohol use in logistic regression models

Table 3

Distributions of the *BRCA2* (rs15869) and *BRIP1* (rs7213430) combined genotypes between the SCCHN cases and controls

Combined genotypes	Cases, *n* (%)	Controls, *n* (%)	Adjusted OR (95 % CI)[a]	*P* [a]
Ordinal[b]				
0	833 (77.3)	882 (81.7)	1.00 (reference)	
1	236 (21.9)	191 (17.7)	1.32 (1.06–1.65)	0.013
2	9 (0.8)	6 (0.6)	1.51 (0.51–4.47)	0.455
P_{trend}				0.040
Dichotomized				
0	833 (77.3)	882 (81.7)	1.00 (reference)	
1–2	245 (22.7)	197 (18.3)	1.33 (1.07–1.65)	0.010

[a] Adjusted by age, sex, smoking status and alcohol use in logistic regression models

[b] Numbers of the observed risk genotypes: CC for *BRCA2* rs15869 and GG for *BRIP1* rs7213430

Table 4

Stratified analysis for associations between *BRCA2* (rs15869) and *BRIP1* (rs7213430) variant genotypes and risk of SCCHN

Varibles	*BRCA2* rs15869 (case/control)		Adjusted OR[a] (95% CI)	*BRIP1* rs7213430 (case/control)		Adjusted OR[a] (95 % CI)	Combined effect of risk genotypes[b]		Adjusted OR[a] (95% CI)
	AA/AC	CC		AA/AG	GG		0	1–2	
All subjects	1031/1053	50/32	1.57 (0.98–2.51)	881/916	205/172	1.26 (1.00–1.58)	833/882	245/197	*1.33 (1.07–1.65)*
Age, year									
≤57(median)	546/547	29/20	1.39 (0.76–2.56)	463/489	117/81	*1.54 (1.12–2.13)*	436/469	139/96	*1.57 (1.16–2.12)*
>57(median)	483/502	21/12	1.73 (0.80–3.77)	418/427	88/91	1.01 (0.72–1.42)	397/413	106/101	1.10 (0.80–1.53)
Gender									
Females	252/248	16/8	1.76 (0.69–4.47)	213/214	56/43	1.30 (0.81–2.08)	198/206	69/49	1.43 (0.92–2.23)
Males	777/801	34/24	1.45 (0.83–2.53)	668/702	149/129	1.23 (0.94–1.61)	635/676	176/148	*1.28 (1.00–1.65)*
Smoking status									
Never	285/514	16/16	1.80 (0.88–3.66)	244/451	60/82	1.38 (0.95–2.00)	226/435	75/93	*1.58 (1.12–2.23)*
Former	357/384	12/10	1.23 (0.52–2.89)	305/335	67/62	1.19 (0.81–1.74)	291/324	78/70	1.23 (0.86–1.76)
Current	387/151	22/6	1.12 (0.44–2.89)	332/130	78/28	1.19 (0.73–1.95)	316/123	92/34	1.08 (0.68–1.70)
Alcohol status									
Never	282/456	13/10	*2.47 (1.06–5.77)*	232/394	63/78	1.33 (0.92–1.94)	219/382	75/83	*1.57 (1.10–2.24)*
Former	226/169	9/7	0.80 (0.28–2.29)	197/145	41/30	1.04 (0.61–1.78)	188/138	47/37	0.95 (0.58–1.58)
Current	521/424	28/15	1.31 (0.66–2.59)	452/377	101/64	1.32 (0.92–1.89)	426/362	123/77	1.34 (0.96–1.87)
Tumor site									
Oropharynx	528/1049	22/32	1.28 (0.73–2.26)	448/916	105/172	1.29 (0.98–1.70)	425/882	125/197	*1.34 (1.03–1.73)*
Non-oropharynx	501/1049	28/32	1.67 (0.95–2.93)	433/916	100/172	1.22 (0.91–1.62)	408/882	120/197	1.27 (0.96–1.66)
Stage									
I–II	252/1049	14/32	1.80 (0.92–3.55)	219/916	50/172	1.24 (0.86–1.77)	203/882	62/197	1.36 (0.97–1.90)
III–IV	777/1049	36/32	1.45 (0.88–2.39)	662/916	155/172	1.26 (0.99–1.62)	630/882	183/197	*1.31 (1.04–1.65)*

[a] Adjusted by age, sex, smoking status, and alcohol status in a logistic regression model

[b] The number represents the numbers of the observed risk genotypes: CC for BRCA2 rs15869 and GG for BRIP1 rs7213430