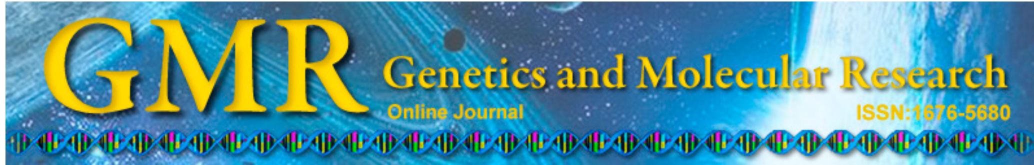

Germline mutations of *DICER1* in Chinese women with *BRCA1/BRCA2*-negative familial breast cancer

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ABSTRACT. Germline mutations in identified breast cancer susceptibility genes account for less than 20% of Chinese familial breast cancers. Dicer is an essential component of the microRNA-producing machinery; germline mutations of *DICER1* have been confirmed in familial pleuropulmonary blastoma, ovarian sex cord-stromal tumors, and other cancers. Low expression of *DICER1* is frequently detected in breast cancer. However, whether germline mutations of *DICER1* occur in familial breast cancers remain unknown. Sixty-five breast cancer probands from *BRCA1/BRCA2*-negative Chinese breast cancer families were screened for germline mutations in *DICER1*. In addition, 100 unrelated healthy females were enrolled as controls. A polymerase

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chain reaction sequencing assay was used to screen for mutations in coding regions and at the exon-intron boundaries of *DICER1*. All variants in introns were evaluated using the NNSplice software to determine the potential splicing effect. A total of 12 germline variants were found, including 11 variants in introns and 1 variant in the 3'-non-coding region. Four variants (IVS8-205 C>T, IVS11+131 delGAAA, IVS16+42 delTA, and IVS19+160 T>C) were novel. Three variants (IVS11+105 C>T, IVS16+42 delTA, and 6095 T>A) may affect splice sites. None of the observed variants appeared to be disease-related, suggesting that germline mutations in *DICER1* are rare or absent in familial breast cancer patients.

Key words: Breast cancer susceptibility; *DICER1*; Germline mutation

INTRODUCTION

The morbidity rate of breast cancer was 42.55 per 100,000 Chinese women in 2009, ranking 1st in cancer incidence and 5th in cancer-related deaths in females (Chen et al., 2013). Breast cancer is frequently related to genetic predisposition, and germline mutations associated with breast cancer have been identified in more than 10 different genes, including *BRCA1*, *BRCA2*, *ATM*, *TP53*, *RAD51C*, and *XRCC2*, among others (Ripperger et al., 2009; Meindl et al., 2010; Park et al., 2012). These genes are mainly involved in pathways critical to genomic integrity. Mutations in these genes in the Chinese population have been confirmed to be at a lower frequency than in other ethnic groups. Mutations in these genes explain less than 20% of familial breast cancer in the Chinese population (Pang et al., 2011; Cao et al., 2010, 2013a,b), indicating that germline mutations in other genes may be involved.

Dicer is an essential member of the RNAase III family, which controls maturation of microRNAs. MicroRNAs regulate the expression of over 30% of genes at the post-transcriptional level. MicroRNAs are transcribed as pri-microRNAs that undergo processing to become pre-microRNAs in the nucleus. Pre-microRNAs are exported to the cytoplasm where they are processed by Dicer into double-strand microRNAs approximately 22 nucleotides in length (Macrae et al., 2006). Increasing evidence indicates that *DICER1* performs an important role in cancer occurrence and development. *DICER1* functions as a haploinsufficient tumor suppressor (Kumar et al., 2009; McCarthy, 2010; Lambertz et al., 2010) and is down-regulated in several cancers, including breast cancer (Karube et al., 2005; Merritt et al., 2008; Dedes et al., 2011; Yan et al., 2012). In 2009, Hill et al. reported that germline mutations in *DICER1* were the primary cause of familial pleuropulmonary blastoma. Recently, germline mutations in *DICER1* were identified in several cancer cases. Moreover, breast cancers in families with *DICER1* germline deleterious mutations have been reported (Slade et al., 2011; Foulkes et al., 2011). Whether *DICER1* is a susceptibility gene involved in familial breast cancer remains unknown.

In this study, we screened the coding regions and exon-intron boundaries of *DICER1* in affected index cases of 65 *BRCA1/BRCA2*-negative familial breast cancers from Eastern China. One hundred healthy females with no personal or family history of cancer were enrolled as healthy controls. All variants were evaluated for potential splicing effects using *in silico* analysis.

MATERIAL AND METHODS

Subjects

All cases were diagnosed from 2007-2010 in the Zhejiang Cancer Hospital. The criterion of familial breast cancer was that patients had at least one 1st- or 2nd-degree relative affected by breast cancer, regardless of age. Family histories for 4-generation pedigrees of all enrolled cases were obtained from medical records and questionnaires, either from the families and/or from the patients. All breast cancer probands were confirmed as negative for *BRCA1/BRCA2* germline mutations by direct sequencing (32 and 40 pairs of primers were used to amplify the entire coding regions and exon-intron boundaries of *BRCA1* and *BRCA2*, respectively). A total of 100 healthy females with no personal and family history of cancer were enrolled as healthy controls from the Zhejiang Cancer Hospital. Written consent was obtained from all participants. This study was approved by the Research and Ethics Committee of Zhejiang Cancer Hospital.

DNA isolation and mutation analysis

Blood samples were collected from all patients and stored in test tubes containing ethylenediamine tetraacetic acid. Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The entire coding regions and exon-intron boundaries of *DICER1* (NM_177438.2) were screened by polymerase chain reaction (PCR). A total of 26 primer pairs (Hill et al., 2009) were synthesized by Invitrogen (Carlsbad, CA, USA). PCR was composed of 10-20 ng genomic DNA, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 1.0X PCR buffer, 0.2 pmol of each primer, and 1.25 U DNA polymerase (TaKaRa, Shiga, Japan) in a 20-mL total volume. The reaction conditions were as follows: initial denaturation at 95°C for 5 min, followed by 14 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 45 s, and extension at 70°C for 45 s, followed by 20 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, and extension at 70°C for 45 s. Finally, synthesized strands were elongated at 72°C for 10 min. The PCR products were evaluated on standard agarose gels before mutation analysis. If no contaminating bands were present, the fragments were sequenced using the BigDye Terminator Cycle Sequencing Kit and the ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). *DICER1* sequences were extracted from the public draft human genome database (NM_177438.2).

Statistical analysis and bioinformatic analysis

Differences in mean ages between breast cancer patients and control subjects were compared by the independent-sample *t*-test (SPSS version 17.0 for Windows; SPSS, Inc., Chicago, IL, USA). P values of <0.05 were considered to be statistically significant. All variants were evaluated using the NNSplice software to determine the potential splicing effect (http://www.fruitfly.org/seq_tools/splice.html).

RESULTS

Patient features

A total of 65 breast cancer patients with 1 or more affected relatives fulfilled the inclusion criteria and were screened for *DICER1* germline mutations. All subjects were from Eastern China and had no *BRCA1/BRCA2* mutations. In our cohort of 65 breast cancer families, there was an average of 2.4 breast cancer cases per family. Age of onset of breast cancer ranged from 22-74 years. There was no statistical significance in the mean age between breast cancer patients and controls (mean age ± standard deviation: 42.8 ± 1.3 *vs* 44.9 ± 0.9, P = 0.15). In the 65 families, in addition to breast cancer, there were 8 stomach cancers, 7 lung cancers, 6 ovarian cancers, 6 colorectal cancers, 5 endometrial cancers, 2 multiple myelomas, 2 cerebral gliomas, 2 liver cancers, 1 bladder cancer, 1 bile duct cancer, 1 osteosarcoma, 1 esophageal cancer, and 1 mediastinal tumor.

DICER1 sequence variants

Screening of index cases of breast cancer families for germline variants in *DICER1* revealed a total of 12 alternations (Table 1). No variants showed changes in the coding of amino acids, including 11 variants in introns and 1 variant in the 3'-non-coding region. Among these, 4 variants (IVS8-205 C>T, IVS11+131 delGAAA, IVS16+42 delTA, and IVS19+160 T>C) were novel and were not detected in the 100 control subjects.

Table 1. *DICER1* sequence variants detected in *BRCA1/BRCA2*-negative familial breast cancer patients (N = 65) and healthy controls (N = 100).

Nucleotide change[a]	dbSNP ID	Location	Minor allele frequency		Previous report
			Cases (N)	Controls (N)	
IVS8-205 C>T	N/A	Intron8	0.008 (1)	0	No
IVS10-43 G>A	rs74899136	Intron10	0.046 (6)	0.040 (8)	Yes
IVS11+105 C>T	rs2275182	Intron11	0.008 (1)	0	Yes
IVS11+131 delGAAA	N/A	Intron11	0.008 (1)	0	No
IVS12-91 A>G	rs2297730	Intron12	0.415 (54)	0.275 (55)	Yes
IVS16-16 G>A	rs189738689	Intron16	0.008 (1)	0	Yes
IVS16+42 delTA	N/A	Intron16	0.008 (1)	0	No
IVS17-52 A>C	rs142321612	Intron17	0.023 (3)	0.005 (1)	Yes
IVS18+60 T>C	rs147668333	Intron18	0.046 (6)	0.030 (6)	Yes
IVS19+160 T>C	N/A	Intron19	0.008 (1)	0	No
IVS22+9 G>T	rs66997818	Intron22	0 (130)	ND	Yes
6095 T>A	rs13078	3'-UTR	0 (130)	ND	Yes

[a]Numbering based on RefSeq: NC_000014.8 (genomic DNA) and NM_177438.2 (mRNA and protein); N/A: information not available for this variant (not reported in dbSNP); UTR: untranslated region; N: number of alleles; ND: not done.

Bioinformatic analysis

All 11 nucleotide variants were assessed for the potential effects on consensus splice sites. Of the observed changes, IVS11+105 C>T and IVS16+42 delTA may introduce new splice acceptor sites (score 0.60 *vs* 0.44 for the wild-type sequence and 0.78 *vs* 0 for the wild-type sequence, respectively). The 6095 T>A variant was found to introduce a new splice donor site (score 0.67 *vs* 0 for the wild-type sequence). The remaining 8 variants appeared to have no effects on splice sites.

DISCUSSION

DICER1 is essential for mammalian development; *DICER1*-deficient mice died on embryonic day 7.5 because of a lack of detectable multipotent stem cells (Murchison et al., 2005). *DICER1* is a haploinsufficient tumor suppressor. Unlike the tumor suppressor genes such as *RB1* (Knudson, 2001), heterozygous loss of *DICER1* occurs more frequently in tumors, and homozygous loss of this gene results in inhibition of tumorigenesis (Kumar et al., 2009; Lambertz et al., 2010). However, Heravi-Moussavi et al. (2012) reported that an inactivating mutation, regardless of whether the mutation is germline or somatic, is accompanied by a second hit in the RNase IIIb domain of *DICER1* in nonepithelial ovarian cancers, particularly at nucleotides encoding amino acids Asp1709, but also at those encoding Glu1813. These mutations in *DICER1* were found to result in the complete loss of 5¢ mature microRNAs. However, 3¢ mature microRNA expression was only partially reduced. Moreover, a study by Kim et al. (2012) showed that complete *DICER1* loss combined with complete *PTEN* loss in the same Müllerian-derived cells (double-knockout) led to high-grade serous carcinoma of the Fallopian tube. These results challenged the previous view that *DICER1* is a haploinsufficient tumor suppressor. Thus, the classical 2-hit model may be accurate (Choong et al., 2012).

Heterozygous loss of *DICER1* globally decreases microRNA expression, followed by regulation of mRNA expression at the post-transcriptional level. A series of events is involved in tumorigenesis. However, the identity and number of events are unknown. The risk of tumors in *DICER1* mutation carriers is low, and most carriers of mutations do not develop tumors (Slade et al., 2011). Germline mutations in *DICER1* are associated with several tumors and other diseases known as *DICER1* syndrome (Slade et al., 2011). The major tumors and other diseases occurring in *DICER1* syndrome include familial pleuropulmonary blastoma (Hill et al., 2009; Slade et al., 2011; Foulkes et al., 2011; Schultz et al., 2011), familial cystic nephroma (Bahubeshi et al., 2010; Slade et al., 2011; Foulkes et al., 2011), familial multinodual goiter (Foulkes et al., 2011; Rio Frio et al., 2011), and ovarian sex cord-stromal tumor (Slade et al., 2011; Foulkes et al., 2011; Schultz et al., 2011; Rio Frio et al., 2011). In addition, embryonal rhabdomyosarcomas, Wilms tumor, medulloblastoma/infratentorial primitive neuroectodermal tumors, intraocular medulloepithelioma, seminoma, uterine cervix embryonal rhabdomyosarcomas, cervical primitive neuroectodermal tumors, and juvenile intestinal polyps have been identified (Slade et al., 2011; Foulkes et al., 2011; Doros et al., 2012).

Interestingly, Slade et al. (2011) reported that a woman carrying the *DICER1* c.5122_5128delGGAGATG mutation subsequently developed a Sertoli-Leydig tumor at 21 years, melanoma at 50 years, endometrial cancer at 62 years, and breast cancer at 68 years. The c.946_962del (p.W316fs*15) truncated mutation of *DICER1* was confirmed in the breast cancer cell line ZR-75-30. Moreover, Foulkes et al. (2011) found that breast cancer occurred in a family with *DICER1* germline mutations.

In this study, we screened the entire coding regions and exon-intron boundaries of *DICER1* in 65 *BRCA1/BRCA2*-negative Chinese women with familial breast cancer. Although several novel variants were identified, these variants are unlikely to be pathogenic. The use of a PCR sequencing assay ensured that all mutations could be detected. However, our sample size was small. In addition, the frequency of *DICER1* deleterious germline mutations is very low in Chinese women with familial breast cancer; thus, it is possible that the sample size was not large enough to include all possible mutations.

Three variants (IVS11+105 C>T, IVS16+42 delTA, and 6095 T>A) may exert det-

rimental effects on the splicing or transcriptional stability of *DICER1*. IVS16+42 delTA was found in an index patient, but not in the control population or public database, suggesting that this polymorphism is rare or a mutation. IVS11+105 C>T (rs2275182) and 6095 T>A (rs13078) were defined as single-nucleotide polymorphisms (SNPs) in a database. Minor allele frequencies of the 2 SNPs were 0.008% and 0, respectively, in our patient group. Jiang et al. (2013) evaluated the SNPs (rs13078) in the *DICER1* gene and the risk of breast cancer in Chinese women. Their study included 878 breast cancer patients and 900 controls. The minor allele frequencies of rs13078 were both 4.8% in the breast cancer patient group and control group. These results indicated that rs13078 in *DICER1* is not associated with breast cancer risk.

In summary, we analyzed the sequence of *DICER1* in 65 Chinese women with *BRCA1/BRCA2*-negative familial breast cancer. Our data suggest that mutations that predispose individuals to breast cancer are either very rare or absent in the coding region of *DICER1*. Whether large genomic rearrangements or mutations in transcriptional regulatory regions are located further away from the coding region, representing alternative and more typical methods of dysregulating *DICER1* function, require further analysis. However, it is possible that germline mutations in this gene are poorly tolerated, and mostly predispose individuals to rare tumors. This is the first study exploring the potential involvement of *DICER1* in hereditary predisposition to breast cancer.

Conflicts of interest

The authors declare no conflict of interest.

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REFERENCES

Bahubeshi A, Bal N, Rio Frio T, Hamel N, et al. (2010). Germline DICER1 mutations and familial cystic nephroma. *J. Med. Genet*. 47: 863-866.

Cao AY, Hu Z and Shao ZM (2010). Mutation screening of breast cancer susceptibility genes in Chinese high-risk families: the results will develop the genetic testing strategy in China. *Breast Cancer Res. Treat*. 120: 271-272.

Cao W, Wang X and Li JC (2013a). Hereditary breast cancer in the Han Chinese population. *J. Epidemiol*. 23: 75-84.

Cao W, Wang X, Gao Y, Yang H, et al. (2013b). BRCA1 germ-line mutations and tumor characteristics in eastern Chinese women with familial breast cancer. *Anat. Rec*. 296: 273-278.

Chen W, Zheng R, Zhang S, Zhao P, et al. (2013). Report of incidence and mortality in China cancer registries, 2009. *Chin. J. Cancer Res*. 25: 10-21.

Choong CS, Priest JR and Foulkes WD (2012). Exploring the endocrine manifestations of DICER1 mutations. *Trends Mol. Med*. 18: 503-505.

Dedes KJ, Natrajan R, Lambros MB, Geyer FC, et al. (2011). Down-regulation of the miRNA master regulators Drosha and Dicer is associated with specific subgroups of breast cancer. *Eur. J. Cancer* 47: 138-150.

Doros L, Yang J, Dehner L, Rossi CT, et al. (2012). DICER1 mutations in embryonal rhabdomyosarcomas from children with and without familial PPB-tumor predisposition syndrome. *Pediatr. Blood Cancer* 59: 558-560.

Foulkes WD, Bahubeshi A, Hamel N, Pasini B, et al. (2011). Extending the phenotypes associated with DICER1 mutations. *Hum. Mutat*. 32: 1381-1384.

Heravi-Moussavi A, Anglesio MS, Cheng SW, Senz J, et al. (2012). Recurrent somatic DICER1 mutations in nonepithelial

ovarian cancers. *N*. *Engl*. *J*. *Med*. 366: 234-242.
Hill DA, Ivanovich J, Priest JR, Gurnett CA, et al. (2009). DICER1 mutations in familial pleuropulmonary blastoma. *Science* 325: 965.
Jiang Y, Chen J, Wu J, Hu Z, et al. (2013). Evaluation of genetic variants in microRNA biosynthesis genes and risk of breast cancer in Chinese women. *Int*. *J*. *Cancer* 133: 2216-2224.
Karube Y, Tanaka H, Osada H, Tomida S, et al. (2005). Reduced expression of Dicer associated with poor prognosis in lung cancer patients. *Cancer Sci*. 96: 111-115.
Kim J, Coffey DM, Creighton CJ, Yu Z, et al. (2012). High-grade serous ovarian cancer arises from Fallopian tube in a mouse model. *Proc*. *Natl*. *Acad*. *Sci*. *U*. *S*. *A*. 109: 3921-3926.
Knudson AG (2001). Two genetic hits (more or less) to cancer. *Nat*. *Rev*. *Cancer* 1: 157-162.
Kumar MS, Pester RE, Chen CY, Lane K, et al. (2009). Dicer1 functions as a haploinsufficient tumor suppressor. *Genes Dev*. 23: 2700-2704.
Lambertz I, Nittner D, Mestdagh P, Denecker G, et al. (2010). Monoallelic but not biallelic loss of Dicer1 promotes tumorigenesis *in vivo*. *Cell Death Differ*. 17: 633-641.
Macrae IJ, Zhou K, Li F, Repic A, et al. (2006). Structural basis for double-stranded RNA processing by Dicer. *Science* 311: 195-198.
McCarthy N (2010). Cancer: Small losses, big gains with microRNAs. *Nat*. *Rev*. *Genet*. 11: 8.
Meindl A, Hellebrand H, Wiek C, Erven V, et al. (2010). Germline mutations in breast and ovarian cancer pedigrees establish RAD51C as a human cancer susceptibility gene. *Nat*. *Genet*. 42: 410-414.
Merritt WM, Lin YG, Han LY, Kamat AA, et al. (2008). Dicer, Drosha, and outcomes in patients with ovarian cancer. *N*. *Engl*. *J*. *Med*. 359: 2641-2650.
Murchison EP, Partridge JF, Tam OH, Cheloufi S, et al. (2005). Characterization of Dicer-deficient murine embryonic stem cells. *Proc*. *Natl*. *Acad*. *Sci*. *U*. *S*. *A*. 102: 12135-12140.
Pang Z, Yan L, Zhang J, Ouyang T, et al. (2011). RAD51C germline mutations in Chinese women with familial breast cancer. *Breast Cancer Res*. *Treat*. 129: 1019-1020.
Park DJ, Lesueur F, Nguyen-Dumont T, Pertesi M, et al. (2012). Rare mutations in XRCC2 increase the risk of breast cancer. *Am*. *J*. *Hum*. *Genet*. 90: 734-739.
Rio Frio T, Bahubeshi A, Kanellopoulou C, Hamel N, et al. (2011). DICER1 mutations in familial multinodular goiter with and without ovarian Sertoli-Leydig cell tumors. *JAMA* 305: 68-77.
Ripperger T, Gadzicki D, Meindl A and Schlegelberger B (2009). Breast cancer susceptibility: current knowledge and implications for genetic counselling. *Eur*. *J*. *Hum*. *Genet*. 17: 722-731.
Schultz KA, Pacheco MC, Yang J, Williams GM, et al. (2011). Ovarian sex cord-stromal tumors, pleuropulmonary blastoma and DICER1 mutations: a report from International Pleuropulmonary Blastoma Registry. *Gynecol*. *Oncol*. 122: 246-250.
Slade I, Bacchelli C, Davies H, Murray A, et al. (2011). DICER1 syndrome: clarifying the diagnosis, clinical features and management implications of a pleiotropic tumor predisposition syndrome. *J*. *Med*. *Genet*. 48: 273-278.
Yan M, Huang HY, Wang T, Wan Y, et al. (2012). Dysregulated expression of dicer and drosha in breast cancer. *Pathol*. *Oncol*. *Res*. 18: 343-348.