BRCC3 mutations in myeloid neoplasms

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ABSTRACT

Next generation sequencing technologies have provided insights into the molecular heterogeneity of various myeloid neoplasms, revealing previously unknown somatic genetic events. In our cohort of 1444 cases analyzed by next generation sequencing, somatic mutations in the gene *BRCA1-BRCA2-containing complex 3* (*BRCC3*) were identified in 28 cases (1.9%). BRCC3 is a member of the JAMM/MPN+ family of zinc metalloproteases capable of cleaving Lys-63 linked polyubiquitin chains, and is implicated in DNA repair. The mutations were located throughout its coding region. The average variant allelic frequency of *BRCC3* mutations was 30.1%, and by a serial sample analysis at two different time points a *BRCC3* mutation was already identified in the initial stage of a myelodysplastic syndrome. *BRCC3* mutations commonly occurred in nonsense (n=12), frameshift (n=4), and splice site (n=5) configurations. Due to the marginal male dominance (odds ratio; 2.00, 0.84-4.73) of *BRCC3* mutations, the majority of mutations (n=23; 82%) were hemizygous. Phenotypically, *BRCC3* mutations were frequently observed in myelodysplastic syndromes and myelodysplastic/myeloproliferative neoplasms and associated with -Y abnormality (odds ratio; 3.70, 1.25-11.0). Clinically, *BRCC3* mutations were also related to higher age (P=0.01), although prognosis was not affected. Knockdown of *Brcc3* gene expression in murine bone marrow lineage negative, Sca1 positive, c-kit positive cells resulted in 2-fold more colony formation and modest differentiation defect. Thus, *BRCC3* likely plays a role as tumor-associated gene in myelodysplastic syndromes and myelodysplastic/myeloproliferative neoplasms.

Introduction

Myelodysplastic syndromes (MDS) are characterized by clonal hematopoiesis, bone marrow failure, a propensity for progression to acute myeloid leukemia (AML) and a variety of molecular abnormalities, including chromosomal aberrations and somatic mutations.[1] In recent years, a large number of somatic mutations affecting new classes of genes have been identified in MDS and related disorders, providing clues to the molecular pathogenesis of these diseases.[2-7] These mutational events can be divided into those that are secondary and acquired during disease progression, and those that are founder in nature.[8] Improved identification of genomic defects has substantiated the view that clinical disease heterogeneity is related to patho-molecular diversity. Clinically, evaluation of somatic defects in MDS may improve diagnosis, accuracy of prognoses, and treatments, i.e. may have implications.

Several novel classes of genes frequently affected by somatic mutations have been found in MDS, including genes involved in cohesin complexes[9] and spliceosomes,[4,10] genes related to methylation[11] and genes of novel receptor tyrosine kinases.[12,13] Because MDS and associated secondary AML (sAML) are diseases of the elderly, accumulation of mutations and alterations arising from DNA damage has been implicated in disease pathogenesis. Consequently, DNA repair defects may play important roles in maintenance of chromosomal integrity and predisposition to secondary molecular defects. Somatic mutations in DNA repair genes such as *TP53* have thus been sought in cancers.[14,15]

Using unbiased sequencing approaches to identify molecular abnormalities in MDS, we probed the mutational status of a number of DNA repair genes. One of these, *BRCA1-BRCA2-containing complex 3* (*BRCC3*), is a member of the JAMM/MPN+ family of zinc metalloproteases capable of cleaving Lys-63 linked polyubiquitin chains.[16] BRCC3 codes for a component of two complexes, BRCA1-A and BRISC. Located in the nucleus, the BRCA1-A complex consists of

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UIMC1, FAM175A, BABAM1, BRE, BARD1, BRCC3 and BRCA1 and participates in DNA double-strand break (DSB) repair.[17] DSBs are associated with genomic instability, cell death[18] and carcinogenesis.[19] To repair DSBs, cells use both homologous recombination (HR), which uses sister-chromatid alleles as templates in late S and G_2, and non-homologous end joining (NHEJ) which can operate in all phases of the cell cycle but often leaves small deletions, possibly gene inactivating at the site of repair.[20] The BRISC complex comprises the following proteins: FAM175B/ABRO1, BRCC3/BRCC36, BRE/BRCC45 and MERIT40/NBA1.

Here, we report that somatic defects of BRCC3 are acquired in various myeloid neoplasms and have consequences at the cellular, and perhaps the clinical level.

Methods

Patients

Bone marrow aspirates or blood were collected from the patients with various myeloid neoplasms seen at the Cleveland Clinic, University of Tokyo and Münchner Leukämielabor GmbH (MLL) (*Online Supplementary Table S1*). Informed consent for sample collection was obtained according to protocols approved by the institutional review boards in accordance with the Declaration of Helsinki. Diagnoses were confirmed according to 2008 World Health Organization classification criteria.[21] A total of 1778 patients were enrolled in this study (*Online Supplementary Table S1*).

Next generation sequencing

Whole exome sequencing (WES) was performed as previously reported.[4,6] Briefly, tumor DNAs were extracted from patients' bone marrow cells. For germ-line controls, DNA was obtained from paired $CD3^+$ T cells. For targeted detection of sequence alterations, confirmation of WES and assessment of variant allelic frequency (VAF), we applied deep sequencing to targeted exons, as previously described.[6,22] Briefly, each targeted exon was amplified with each pair of primers, generating 200bp fragments on average. These amplicons were subjected to massive parallel sequencing (Illumina, San Diego, CA, USA) using TruSeq custom primers (Illumina) and SureSelect (Agilent, Santa Clara, CA, USA) with paired-end reads, according to the manufacture's instruction. Some of these procedures were followed by confirmation using Sanger sequencing, as previously described.[13] Annotations by NCBI reference numbers of the genes affected by somatic mutations are summarized in *Online Supplementary Table S2*.

Cytogenetics and single nucleotide polymorphism-array analyses

Technical details about sample processing and data analyses for single nucleotide polymorphism-array (SNP-A) have been previously described.[23] Affymetrix 250K and 6.0 Kit (Affymetrix) were used. Germ-line copy number variants in our internal database or in a publicly available database (Database of Genomic Variants) (*http://dgv.tcag.ca/dgv/app/home*) were considered non-somatic variants and excluded. Results were analyzed with CNAG (v.3.0)[24] or Genotyping Console (Affymetrix). All other lesions were confirmed as somatic or germ-line by analysis of CD3-sorted cells.

ShRNA knockdown and colony formation assay of LSK cells

Lineage-negative, Sca1-positive, c-kit-positive (LSK) cells were purified from C57BL/6 mice, as previously described.[25] LSK cells were infected with pLKO.1 lentivirus containing shRNAs targeting *Brcc3* (Targeting sequences: shRNA, 5'-CCCACCCT-CATATAACTGTTT-3') or negative control shRNA (Sigma, SHC002). Lentiviral infections were performed twice by spinoculation. Colony formation assays were performed after another 24 h using $2x10^4$ cells on IMDM methylcellulose medium supplemented with 15% horse serum, mouse SCF (100 ng/mL), IL-6 (6 ng/mL), IL-3 (3 ng/mL) and puromycin (2 μg/mL). Colony numbers were counted after seven days.

Statistical analysis

Overall survival was measured from the day of initial sampling to death from any cause (patients lost to follow up were censored) or last follow up and was summarized using Kaplan-Meier plots and analyzed using the Cox proportional hazard model. Results are for data collected as of May 2013. Pair-wise comparisons were performed by Wilcoxon test for continuous variables and by two-sided Fisher exact for categorical variables. Significance was determined at a two-sided alpha level of 0.05, except for *P* values in multiple comparisons, for which Bonferroni correction was applied. Analyses were performed using JMP10 (SAS Inc.).

Results

Identification of BRCC3 mutations in myeloid neoplasms

In our cohort of 149 cases analyzed by WES, 2 patients (1.3%) with refractory cytopenia with multilineage dysplasia (RCMD) and chronic myelomonocytic leukemia-1 (CMML-1) revealed 2 somatic recurrent *BRCC3* mutations (c.C19T, p.Q7X). The somatic nature of these mutations was confirmed by Sanger and targeted deep DNA sequencing (Figure 1). When we expanded our study to the larger cohort for targeted deep sequencing (n=1295), 26 *BRCC3* mutations were identified (2.0%), 11 with refractory anemia with excess blasts (RAEB), 9 with RCMD, 1 with refractory anemia, 1 with refractory anemia with ring sideroblasts, 1 with isolated 5q syndrome, 1 with CMML, 1 with RARS associated with thrombocytosis (RARS-T), and 1 with primary AML (Table 1). Four canonical mutations occurred in exon 1 (p.Q7X), 3 in exon 4 (p.R81X) and 2 in exon 5 (p.W120X). Thus, 28 (1.9%) of our 1444 patients with various myeloid neoplasms exhibited *BRCC3* mutations.

Genetic significance of BRCC3 mutations

The 28 *BRCC3* mutations occurred in nonsense (n=12; 42.9%), missense (n=6; 21.4%), splice site (n=5; 17.9%), frameshift (n=4; 14.3%), and non-frameshift deletion (n=1; 3.6%) forms. Twenty-three (82.1%) were hemizygous due to either male sex or a deletion of chromosome X (-X) (*BRCC3* is located on chromosome X). Evaluation of deep sequencing demonstrated that the mean value of variant allelic frequency of *BRCC3* mutations was 30.1% (range 91.7-3.5). In one of these index cases, deep sequencing at two time points (Case #1 in Table 1) indicated that the *BRCC3* mutation was present at RCMD diagnosis as well as throughout RAEB evolution (20 months later) (Figure 2A). This suggests that the *BRCC3* mutation may have been an early event. By additional analyses on concomitant mutations of other functionally important genes in the cases with *BRCC3* mutations, *TET2* and *SF3B1* mutations were observed most frequently among mutations of 23 genes detected (Figure 2B and C). SNP-A

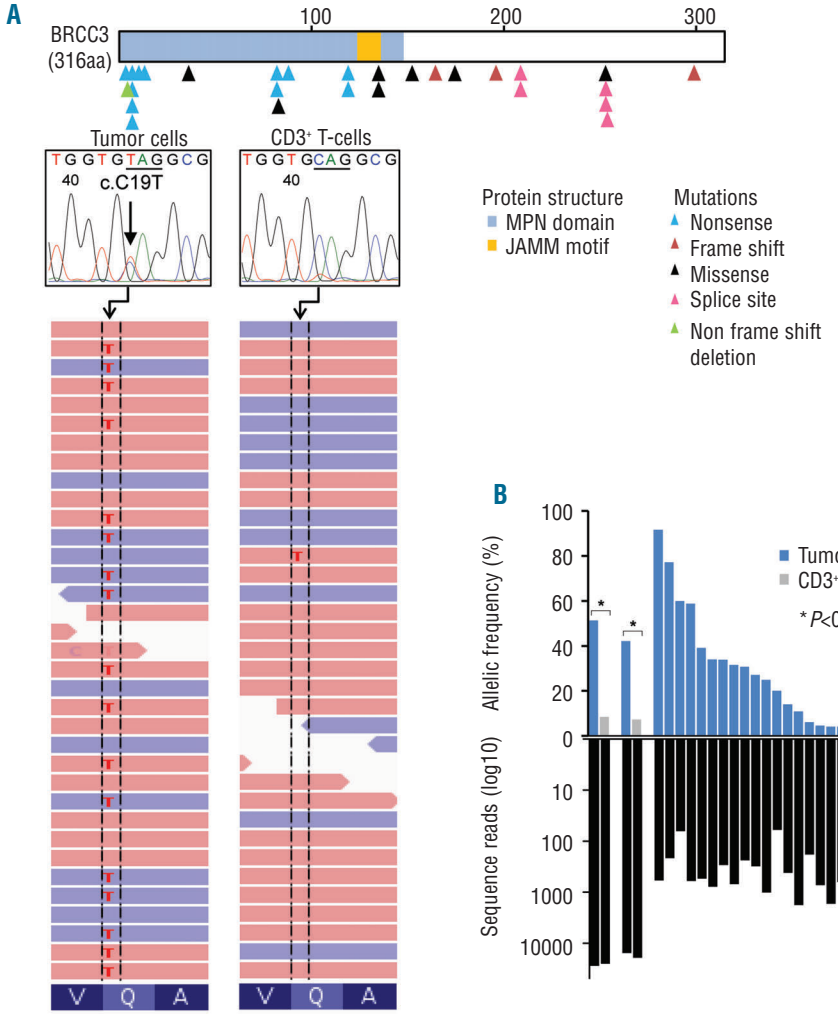

Figure 1. Somatic *BRCC3* mutations as detected by next generation sequencing and Sanger sequencing. (A) Distribution of *BRCC3* mutations identified in 28 out of 1444 myeloid neoplasms. Blue, red, black, pink, and green triangles indicate nonsense, frame shift, missense, splice site, and non-frame shift deletion mutations. On the right, a representative mutation (c.C19T, indicated by an arrow) confirmed by whole exome sequencing and Sanger sequencing. (B) (Top) Allelic frequencies in paired bone marrow and CD3+ T-cell samples (n=2) and not paired (n=23) samples as measured by deep sequencing. (Bottom) Depth of coverage of independent reads.

karyotyping, deletions involving the *BRCC3* locus (Xq28) were detected in 7 out of 677 (1.0%) patients. For *BRCC3* located on chromosome X, expression was assessed by comparison to sex-matched controls. Due to the small sample size, the impact of somatic deletion of chromosome X on *BRCC3* expression could not be resolved (*Online Supplementary Figure S1*). In fact, in our cohort, there was no significant difference in *BRCC3* expression between males and females (*Online Supplementary Figure S2*). In various hematopoietic cells, analysis of mRNA showed *BRCC3* globally expressed in myeloid, erythroid and lymphoid cells (*Online Supplementary Figure S3*).

Clinical phenotype of the cases with BRCC3 *mutations*

BRCC3 mutations were associated with higher age in the whole cohort [mean±standard deviation, 67±13 in wild-type (WT) and 72±10 in mutants; *P*=0.01]. There was a marginally significant male dominance of mutation frequency [odds ratio (OR) = 2.00; 95% confidence interval (CI) 0.84-4.73] (Table 2). With regard to cytogenetics, mutations of this gene were associated with -Y abnormality (OR; 3.70, 95%CI; 1.25-11.0; *P*=0.034), while all other karyotype abnormalities showed similar distributions between WT and mutant cases (Table 2). Phenotypically, *BRCC3* mutations were identified in the low- (13 of 652; 2.0%) and high- (11 of 427; 2.6%) risk MDS groups and in myelodysplastic/myeloproliferative neoplasms (MDS/MPN) (3 of 94; 3.2%), but not in any sAML and myeloproliferative neoplasms (MPN) cases (Table 2). *BRCC3* mutations had no impact on overall survival (*Online Supplementary Figure S4*).

Cellular consequences of BRCC3 lesions

Frequently, *BRCC3* mutations detected in our cohort occurred in nonsense and frameshift configurations associated with myelodysplastic phenotypes. This suggests that *BRCC3* is a candidate tumor suppressor gene in MDS. To clarify the functional consequences of *BRCC3* defects, lentiviral shRNA mediated knockdown of Brcc3 expression was performed in mouse lineage-Sca-1+c-kit+ (LSK) cells, which are enriched for hematopoietic stem and early progenitor cells. Selection with puromycin for 24 h resulted in a pure population (>95%) of transduced cells. *Brcc3* knockdown was confirmed by Western blotting analysis at 96 h after infection with a *Brcc3*-specific lentiviral shRNA. Colony formations significantly increased in LSK cells with *Brcc3* knockdown compared to cells infected with control lentiviral shRNA (*P*<0.001) (Figure 3). *Brcc3* knockdown also resulted in moderate differentiation block (Figure 3), which was confirmed by flow cytometry analyses showing increased expression of precursor marker c-Kit and reduced expression of differentiation marker Gr-1 with *Brcc3* knockdown (*Online Supplementary Figure S5*).

Genetic defects of other component of BRCA1-A and BRISC complex

BRCC3 is a component of the BRCA1-A and BRISC complexes. We, therefore, analyzed other genes in these complexes in 149 cases and observed mutations in 3 patients (2.7%) (*Online Supplementary Table S3*). The mutations were in the genes *UIMC1, FAM175A* and *BABAM1*. By SNP-array karyotyping, deletions in BRCA1-A and BRISC component genes were found in 21 of 677 (3.1%) patients. *UIMC1* deletions (5q35.2) were identified in 11 patients, *FAM175A* deletions (4q21.23) were present in 8 (*Online Supplementary Figure S6*) and those involving *FAM175B* and *BABAM1* loci were each present in one patient (*data not shown*). Out of these genes affected in myeloid neoplasms, frequently deleted *UIMC1* showed haploinsufficient expression ($P<0.05$) (*Online Supplementary Figure S7*).

Discussion

Here, we report the discovery of somatic mutations in the *BRCC3* gene participating in the BRCA1-A and BRISC complexes in myeloid neoplasms. After the initial discovery of nonsense mutations in this gene by WES, our cohort for target deep sequencing was expanded and 28 mutations were discovered in various myeloid neoplasms. These mutations were frequently hemizygous and present in nonsense and frameshift forms. *BRCC3* mutations were more common in MDS and MDS/MPN compared to primary AML and MPN, and were cytogenetically associated with -Y abnormality. Deep sequencing analysis of clonal architecture in serial samples indicated that *BRCC3* mutations may constitute founder events, and *Brcc3* knockdown in murine LSK cells resulted in approximately doubling clonogenic cells and modest differentiation block of hematopoietic progenitors transfected with *Brcc3* shRNA. The pro-leukemogenic effects of *BRCC3* mutations might be related to the impact of these lesions on tumor suppressive potential.

In addition to *BRCC3*, other genes encoding components of BRCA1-A and BRISC complexes were also affected by either somatic mutations or deletions. These components assemble to play a role in DSBs repair.[17] For this process, canonical non-homologous end joining (C-NHEJ) is initiated by binding of the Ku70/80 heterodimer to DSB free ends; DNA-bound Ku protects the DSB free ends from nuclease digestion[26] while it recruits DNA-PK cat-

Table 1. Characteristics of patients with *BRCC3* mutations.

Case	Age (years)	Sex	Diagnosis*	Cytogenetics	BRCC3 (NM_024332) mutations	
					Substitution	Zygosity
1	61	F	RCMD	46,XX,add(15)(p11.1),add(22)(p11.2)[3]/47,idem,+19 [19].	c.C19T: p.Q7X	Hetero
2	78	M	CMML-1	46,XY[20]	c.C19T: p.Q7X	Hemi
3	69	M	CMML-1	46,XY[20]	c.C241T: p.R81X	Hemi
4	71	F	pAML	Complex (including del(Xq))	c.C241T: p.R81X	Hemi
5	44	M	RAEB-1	45,XY,-7[21],del(12)(q24q24),add(14)(q32q32)	c.A521G: p.Q174R	Hemi
6	75	M	RAEB-1	46,XY,der(4)t(4;16)(q23;q23),der(16)t(4;16)(q31;q23)	Splice site	Hemi
7	72	F	RCMD	46,XX,i(17)(q10)	c.C19T: p.Q7X	Hetero
8	68	M	RCMD	46,XY	c.C10T: p.Q4X	Hemi
9	81	M	RAEB-1	45,X,-Y	Splice site	Hemi
10	83	M	RARS-T	46,XY	c.489delA: p.T163fs	Hemi
11	59	M	RAEB-2	46,XY	c.C28T: p.Q10X	Hemi
12	70	F	RCMD	46,XX	c.T405A: p.D135E	Hetero
13	76	F	RAEB-1	46,XX	c.T112C: p.C38R	Hetero
14	77	M	RAEB-1	46,XY	c.587_588del: p.Q196fs	Hemi
15	80	M	RAEB-1	46,XY	c.T405A: p.D135E	Hemi
16	77	F	RA	45,X,-X	Splice site	Hemi
17	84	M	RCMD	46,XY	c.G360A: p.W120X	Hemi
18	86	M	RCMD	46,XY	Splice site	Hemi
19	75	M	RAEB-2	46,XY	c.15_23del: p.5_8del	Hemi
20	78	M	RCMD	46,XY	c.C265T: p.R89X	Hemi
21	60	M	RCMD	46,XY,del(20)(q11)	c.T455A: p.L152H	Hemi
22	77	M	5q-	46,XY,del(5)(q14q34)	Splice site	Hemi
23	47	M	RAEB-2	46,XY	c.G43T: p.E15X	Hemi
24	70	M	RARS	45,X,-Y,der(16)t(3;16)(q22;q24)	c.C19T: p.Q7X	Hemi
25	68	F	RCMD	46,XX	c.G755A: p.S252N	Hetero
26	84	M	RAEB-2	45,X,-Y	c.G245A: p.R82H	Hemi
27	83	M	RAEB-2	45,X,-Y	c.889delC: p.H297fs	Hemi
28	78	M	RCMD	46,XY	c.G360A: p.W120X	Hemi

**Diagnoses are according to WHO 2008 classification. F: female; M: male; VAF: variant allelic frequency; NA: not analyzed.*

alytic subunit (DNA-PKcs) to the DSB. The resultant DNA-PK complex activates DNA-PK's kinase activity[27,28] which phosphorylates H2AX to form γH2AX. It also facilitates the recruitment of a ligation complex, which encompasses DNA LigIV and XRCC4 among other proteins. Compromised BRCC3 function in DSB repair, which is presumably to remove DSB repair proteins by degradation during DSB resolution, may cause an imbalance repair

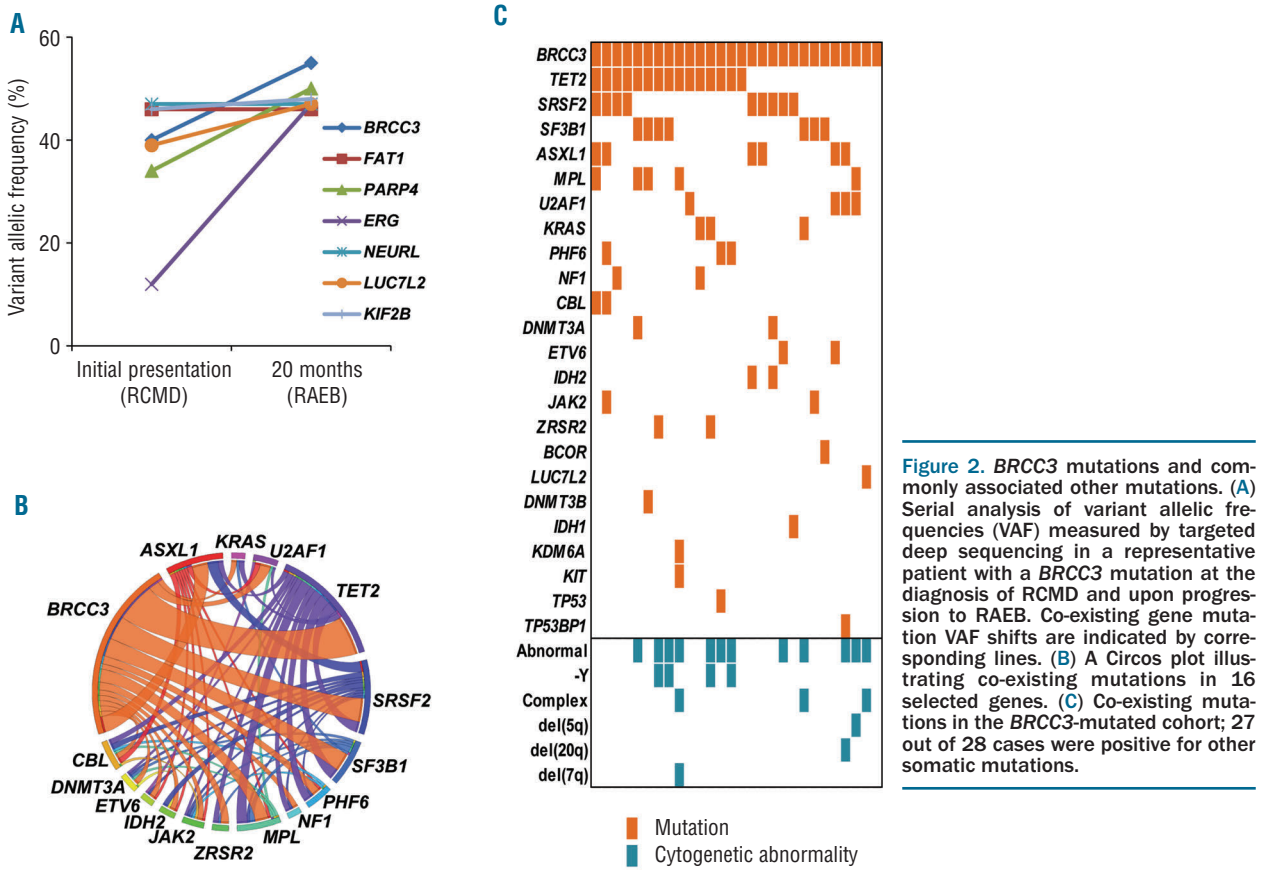

Figure 2. *BRCC3* mutations and commonly associated other mutations. (A) Serial analysis of variant allelic frequencies (VAF) measured by targeted deep sequencing in a representative patient with a *BRCC3* mutation at the diagnosis of RCMD and upon progression to RAEB. Co-existing gene mutation VAF shifts are indicated by corresponding lines. (B) A Circos plot illustrating co-existing mutations in 16 selected genes. (C) Co-existing mutations in the *BRCC3*-mutated cohort; 27 out of 28 cases were positive for other somatic mutations.

Table 2. Clinical characteristics of myeloid malignancies either with or without *BRCC3* mutations.

Variables	Wild-type *BRCC3*	*BRCC3* mutant	Odds ratio (Lower 95%-Upper 95%)	P[a]
N. (%)	1416 (98.1)	28 (1.9)		
Age at study entry (years); mean ± s.d.	67±13	72±10	N/A	**0.01**[b]
Range	18-90	46-86		
Male sex; no. (%)	835 (60)	21 (75)	2.00 (0.84-4.73)	0.12
Bone marrow blasts (%); mean ± s.d.	11±18	7±10	N/A	0.56
Diagnosis; n. (%)				
Low-risk MDS	639 (45.1)	13 (46.4)	1.05 (0.50-2.23)	1.00
High-risk MDS	416 (29.4)	11 (39.3)	1.56 (0.72-3.35)	0.30
sAML	35 (2.4)	0	N/A	1.00
MDS/MPN	92 (6.5)	3 (10.7)	1.73 (0.51-5.83)	0.42
MPN	22 (1.6)	0	N/A	1.00
pAML	209 (14.8)	1 (3.6)	0.21 (0.03-1.58)	0.11
Cytogenetics; n. (%)				
Abnormal	532 (37.8)	12 (42.9)	1.23 (0.58-2.62)	0.69
-5, del (5q)	119 (8.5)	1 (3.6)	0.40 (0.05-2.97)	0.72
-7, del (7q)	79 (5.6)	1 (3.6)	0.62 (0.08-4.64)	1.00
-Y	61 (4.3)	4 (14.3)	**3.70 (1.25-11.0)**	**0.034**
-20, del (20q)	61 (4.3)	1 (7.1)	0.82 (0.11-6.11)	1.00
+8	92 (6.5)	0	N/A	0.25
Complex (≥3)	119 (8.5)	3 (10.7)	1.29 (0.39-4.36)	0.73

[a]*A Fisher's exact test was used to determine the P values, except where otherwise indicated. Statistically significant P values were indicated with bold font.* [b]*A Wilcoxon test was used to calculate the P values.* [c]*A log-rank test was used to calculate the P value. N/A; not applicable.*

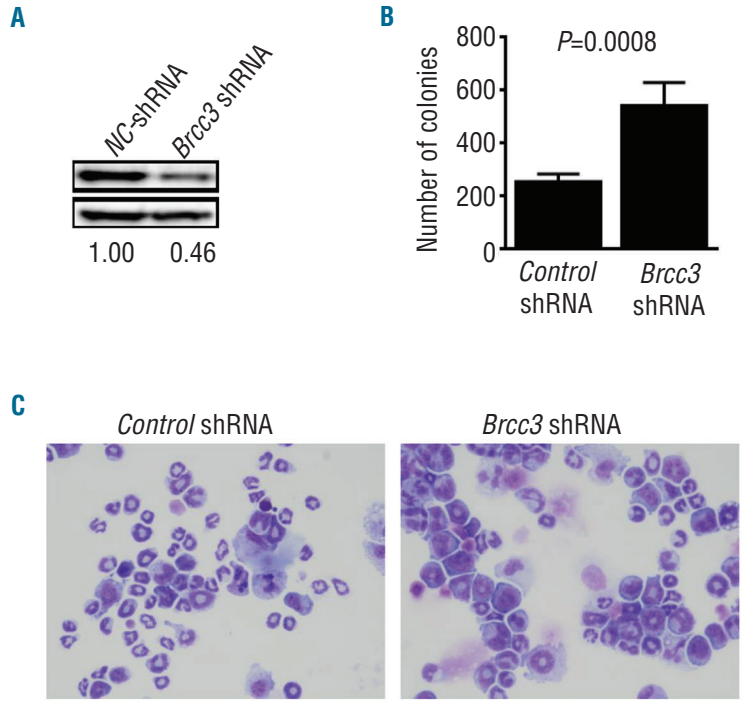

Figure 3. Cellular consequences of *BRCC3* knockdown. (A) Immunoblot analysis of Brcc3 levels in LSK cells at 96 h after infection with lentivirus carrying *Brcc3* -specific or control (NC) shRNA. (B) *Brcc3* knockdown significantly increased colony formation capability of murine lineage$^-$ Sca-1$^+$c-kit$^+$ (LSK) cells. Columns correspond to mean ± SD of colony numbers formed by 5000 puromycin-resistant murine LSK cells in the presence of SCF, IL-3, and IL-6 at 48 h after infection with lentivirus carrying *Brcc3*-specific or control shRNA and subsequent selection with puromycin. (C) Wright-Giemsa staining of LSK cells in the presence of SCF, IL-3, and IL-6 at 7 days after infection with lentivirus carrying *Brcc3*-specific or control shRNA (X200).

proteins globally, and as a result, genomic instability. In our cohort with *BRCC3* mutations, multiple somatic driver mutations and cytogenetic abnormalities were identified, and one gave a cancer cell survival advantage that enabled genomic instability caused by BRCC3.

In analogy to somatic *BRCC3* mutations, acquired mutations of other genes involved in DNA repair machinery have been reported in MDS and related conditions, for example, in *TP53* and *ATM*. TP53-deficient cell lines show delayed γH2AX resolution post-irradiation.[18,29] Similarly, mutant TP53 prevents the MRE11–RAD50–NBS1 complex from phosphorylating ATM, leading to impaired HR.[30] ATM is activated by DSB and signals the cell-cycle checkpoint to slow the passage of cells through the cycle to allow time for DNA repair.[31] Germ-line mutations in DNA repair machinery genes, including *BRCA1* and *BRCA2*, *TP53* and *ATM* convey a strong inherited predisposition to neoplasia.[32-35] However, disease-prone germline variants of *BRCC3* have not been reported so far.

Defects in DNA repair acquired in the malignant clone may create a leukemia cell-specific vulnerability, if it leads to a greater number of residual DSBs 24 h after their induction.[18] Based on this theory, cells with partial loss of *BRCC3* function could be sensitive to BRCC3 inhibition[36] as they will have greater dependence on the remaining WT allele and thus be differentially more stressed by its inactivation. This suggests, however, a broader target, namely, any member of either the BRCA1-A or BRISC complexes might be capable of incapacitating the entire complex.

Knockdown of *Brcc3* in normal hematopoietic cells resulted in the modest increase of colony formation and slightly less differentiation compared to WT experiments, which might support the evidence of the tumorigenic effects of human *BRCC3* mutations. However, shRNA transductions were applied for *Brcc3* knockdown with a single targeting sequence. Off target effects should always be considered when we perform knockdown experiments using a single shRNA. For validation of the effect, we showed the successful reduction of *Brcc3* protein amount by immunoblot to confirm the suppressive effects of shRNA. Further rescue experiments and animal models are essential to authenticate these findings on *BRCC3*-associated leukemogenesis.

In summary, we have found new somatic mutations of *BRCC3* and deletions of other BRCA1-A complex component in MDS and MDS/MPN. These results suggest that *BRCC3* might be a tumor-associated gene in myeloid neoplasms.

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Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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