Characteristics of myeloid sarcoma in mice and patients with *TET2* deficiency

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Abstract. Myeloid sarcoma (MS) carries a poor prognosis, and information on epigenetic modifications in MS is currently limited. In the present study, 214 ten-eleven translocation-2 (*TET2*)$^{-/-}$ mice were successfully constructed. In addition, 436 patients with myelodysplastic syndrome (MDS) and 354 with acute myeloid leukemia (AML) patients were recruited. The incidence of MS in mice and patients with *TET2* deficiency was examined, and the efficiency of hypomethylating agents (HMAs) was also be evaluated. A total of 93% of the *TET2*$^{-/-}$ mice developed myeloid malignancies, 5.5% of which were accompanied by MS (n=11). The survival of these *TET2*$^{-/-}$ mice ranged between 3 and 25 months. No significant difference was observed between the survival of MS and non-MS mice with *TET2* loss (P>0.05). In addition, MS cells were transplantable, and their recipients exhibited myeloproliferative characteristics, such as increased white blood cell counts, monocytosis, low erythrocyte counts and hepatosplenomegaly. Their median survival duration was 94.8 days. In the clinical setting, 9.7% of MDS and 11.6% of AML patients with *TET2* deficiency developed MS, which was higher compared with previous reports (1.5-9.1%). The median age of the MS patients was 44 years old. 5-Aza-2'-deoxycytidine (5-Aza-dC) reduced the incidence of MS in *TET2*$^{-/-}$ mice, and decitabine was a suitable treatment strategy for MS patients. These data indicate that *TET2* deficiency plays a key role in MS and its prognostic significance requires further investigation. HMAs may be a useful treatment for MS patients with *TET2* mutations.

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Introduction

Myeloid malignancies develop when myeloblasts infiltrate extramedullary tissue, and they include myeloid sarcoma (MS) and leukemia cutis (LC) (1). The term MS is often currently applied to any tumor associated with acute leukemia (AL) or myelodysplastic syndrome (MDS) and MS is reported in 2.5-9.1% of acute myeloid leukemia (AML) patients (2-4), and may occur concomitantly, following or rarely preceding the onset of systemic bone marrow leukemia (5). The clinical manifestations of MS are diverse given its various sites of occurrence, with signs and symptoms determined by its specific location and size. Patients may suffer from MS or display molecular alterations prior to the appearance of clinical symptoms.

Similar to myeloid diseases, MS is genetically and epigenetically diverse in nature and often poses a diagnostic challenge and therapeutic dilemma (6). Cytogenetic and molecular biological factors in MS, such as alterations in

chromosome 8, FLT3-ITD and NPM1, were previously investigated (7). A variety of chromosomal disorders and mutations, such as the most commonly reported t(8;21) translocation and 11q23 abnormalities, are associated with a higher incidence of MS both at presentation and at relapse, indicating a poor response and relapse risk (8-11).

To date, the available information on epigenetic modifications and treatments associated with MS has been very limited (12,13). The current guidelines recommend that MS patients should be treated in the same manner as patients with AML. Chemotherapy, radiotherapy and hematopoietic stem cell transplantation (HSCT) are the most common modalities used (14). Hypomethylating agents (HMAs), which reduce the activity of DNA methyltransferase (DNMT) activity in cells and lead to the hypomethylation of DNA, are also a first-line treatment for MDS in all ages and an alternative treatment for patients with AML, resulting in the re-expression of genes necessary to control cellular differentiation and proliferation (15). However, no randomized trials to date have addressed the optimal treatment for MS patients with ten-eleven translocation (*TET*)*2* deficiency.

Members of the ten-eleven translocation (*TET*) gene family, one of the most important families of epigenetic regulatory genes, are key enzymes for DNA demethylation (16). *TET2* is a critical regulator of HSC homeostasis, and its functional impairment leads to myeloid malignancies, such as AML and MDS (17,18). In addition, loss-of-function mutations of *TET2* have been detected in patients with MS (19). Previously published literature has reported decreased *TET2* mRNA levels in MS patients (20), which may indicate the importance of *TET2* in MS. Therefore, epigenetic abnormalities and the role of demethylating drugs in MS should be further investigated.

The aim of the present study was to investigate whether *TET2*$^{-/-}$ mice developed MS with characteristics similar to those of patients with MS, and whether hypomethylating therapy (HMT) is effective in both mouse models and MS patients with *TET2* deficiency.

Materials and methods

Mice and methods. To determine the complete spectrum of MS caused by *TET2* loss *in vivo*, a 2-year follow-up study on a cohort of 214 *TET2*$^{-/-}$ and 67 wild-type (WT) mice was conducted. *TET2* knockout (*TET2*$^{-/-}$) mice were generated as previously described (21). Animal care was performed in accordance with institutional guidelines and was approved by the Institutional Animal Care and Use Committee at the Second Hospital of Tianjin Medical University. Mice were housed in a temperature controlled room (22˚C) under a 12/12 h light/dark cycle and received water and food *ad libitum* throughout the experimental protocol. And MS was diagnosed by examinning blood counts, flow cytometric analyses (Mac1^{+}/Gr1^{+} and high forward scatter myeloid cells) and size of liver and spleen of these mice. Peripheral blood (PB) was collected from the mice by tail vein and subjected to an automated blood count (Hemavet System 950FS, Drew Scientific). For PB smear, smears for Leishman and Giemsa stains, were created from WT and sarcoma blood samples. The slides were reviewed by two expert haematopathologists, based on the staining characteristics such as the nuclear features, cytoplasmic features, degree of granularity of the cytoplasm and other morphological red blood cell (RBC) and white blood cell (WBC) characteristics. For flow cytometric analyses, Mice were killed by cervical dislocation and single-cell suspensions from the liver, uterus, and PB were stained with panels of fluorochrome-conjugated antibodies. Dead cells were excluded by 4,6-diamidino-2-phenylindole staining. Analyses were performed using a BD FACSCanto II or LSRII flow cytometer (BD Biosciences). All data were analyzed by FlowJo7.6 software.

Aza-dC was purchased from Sigma-Aldrich; Merck KGaA and diluted in Dulbecco's PBS (PAN Biotech GmbH). For its use in *in vivo* experiments, fresh stocks of 120 μg/ml 5-Aza-dC were prepared on ice and diluted in PBS to the desired drug dose for each animal. Aliquots were stored for a maximum period of 2 weeks at -80˚C. 5-Aza-dC diluted with PBS was injected intravenously through the tail vein (2 mg/kg) 30 min after unfreezed. An equal volume of PBS was administered to sham and model mice.

Transplantation assay. To evaluate the malignant nature of the abnormally infiltrating MS cells in *TET2*$^{-/-}$ mice, and to determine the transplantability of sarcoma cells, 1x10^{6} bone marrow (BM) cells from WT mice or 1x10^{6} sarcoma cells were injected into sublethally irradiated (800 cGy) recipients (n=5) (Fig. 3A) through the tail veins. Recipient mice became moribund or died 7 months after injection. The mice were analyzed to determine their hematological phenotype and the development of myeloid malignancy. Donor cell chimeras in the PB and the weight of the liver and spleen were examined at the end of the observation period.

Drug treatments. To explore the role of 5-Aza-dC in *TET2*$^{-/-}$ myeloid malignancies, a 6-month follow-up study was conducted on *TET2*$^{-/-}$ mice; the experimental group was treated with 5-Aza-dC (2 mg/kg, n=30), and the control group was not treated (n=51). After 6 months, the survival, hematological parameters and size of the liver and spleen for the mice were analyzed. In addition, mice with sarcoma (n=5) were injected with 5-Aza-dC daily over a period of 4 weeks and followed up for an additional 8 weeks. The mice were monitored daily for abnormal signs. Routinely we check and observe the hematological parameters, weight, appetite, energy and appearance of the 214 mice. Once observing the abnormal hematological parameters or signs, such as extreme weight loss, lack of feeding, grooming, pain, abnormal skin nodules and testicular swelling, we will sacrifice the mice.

Patients. A total of 436 MDS and 354 AML patients with complete follow-up information were recruited to this study between 2001 and 2018. The patients were recruited from several clinical centers: The Second Hospital of Tianjin Medical University, the Oncology Hospital of Tianjin Medical University, the Union Hospital of Tongji Medical College of Huazhong University of Science and Technology and the First Affiliated Hospital of Chongqing Medical University. MDS patients were classified according to the WHO 2016 classification (22), and risk stratification was performed according to the Revised International Prognostic Scoring System (IPSSR; Table I). AML patients were classified into primary and

Table I. Characteristics of patients with MDS (n=436).

Characteristic	No. (%)
Sex	
Male (%)	278 (63.8)
Age, years (range)	48 (15-74)
MS (%)	38 (8.7)
WHO classification[a]	
MDS with single lineage dysplasia	12
MDS with ring sideroblasts (MDS-RS)	15
MDS associated with del(5q)	53
MDS with multilineage dysplasia	223
MDS with excess blasts	162
MDS, unclassifiable	11
IPSS, n (%)	
Low	39 (8.9)
Int-1	278 (63.8)
Int-2	93 (21.3)
High	26 (5.9)
Karyotype risk[b], n (%)	
Good	249 (57.2)
Intermediate	107 (24.5)
Poor	80 (18.3)
IPSS-R	
Very low-risk	15 (3.4)
Low-risk	117 (26.8)
Intermediate	138 (31.6)
High-risk	100 (23.1)
Very high-risk	66 (15.1)

[a]The 2016 revision to the World Health Organization classification of MDS. [b]Karyotype risk: Good: Normal, -Y alone, del(5q) alone, del(20q) alone; Poor: Complex (≥3 abnormalities) or chromosome 7 anomalies; Intermediate: Other abnormalities [This excludes karyotypes t(8;21), inv16, and t(15;17), which are considered to be AML and not MDS]. MDS, myelodysplastic syndrome; IPSS, International Prognostic Scoring System; WHO, World Health Organization.

Table II. Characteristics of patients with AML (n=354).

Characteristic	No. (%)
Sex	
Male (%)	139 (39.3)
Age, years (range)	41 (16-69)
MS (%)	32 (9.0)
Classification	
De novo	286 (80.8)
Secondary	68 (19.2)
Gene mutations	
NPM1	106 (30)
FLT3-ITD	88 (24.8)
CEBPA	47 (13.3)
RUNX1	24 (6.8)
ASXL1	32 (9.0)
TP53	34 (12)
Karyotype risk[a], n (%)	
Good	76 (21.5)
Intermediate	166 (46.9)
Poor	112 (31.6)

[a]Karyotype risk: Good, inv(16), t(8;21), t(16;16), normal with NPM mutation alone; Poor, complex (≥3 abnormalities), -5, -7, 5q, 7q, 11q23 anomalies, normal with FLT3-ITD alone without t(9;11), inv(3), t(3;3), t(6;9), t(9;22). Intermediate, normal, +8 alone, t(9;11) alone, other abnormalities [this excludes karyotypes t(8;21) or inv(16) with C-KIT mutation]. AML, acute myeloid leukemia.

MDS-secondary AML, with examination of chromosomes and cytogenetics (Table II). In particular, MS patients with *TET2* deficiency were closely examined and the effect of decitabine was assessed. All patients provided their written informed consent to genetic analyses and research studies. Informed consent was obtained according to the protocols approved by the Institutional Review Board and in accordance with the principles outlined in the Declaration of Helsinki.

Sample collection, DNA extraction and mutation analysis. BM and PB of patients were collected prior to treatment with HMAs. Following red cell lysis, white blood cells (WBC) were collected from the samples and total DNA was extracted. By using a next-generation sequencing approach, 112 hematological malignancy-associated genes including *TET2* were detected. Testing was confined to somatic mutations. Germline polymorphisms were excluded from analysis, including those previously reported in population databases such as ExAC and dbSNP and identified in >20% of the in-house patient population.

Response evaluation. Treatment responses and adverse events were extracted from medical records. The treatment response measures included complete remission (CR), partial remission (PR), stable disease (SD), hematological improvement, and treatment failure using the IWG 2006 criteria (23). Hematological toxicities were assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0 (24). Overall remission (OR) included CR, PR, and hematological improvement. Overall survival (OS) was measured from the beginning of the trial to the death of the patient from any cause or to the date of the last follow-up for surviving patients. Progression-free survival (PFS) was measured from the beginning of the trial until treatment failure, relapse or death from any cause.

Statistical analysis. **S**tatistical software programs, including SPSS 21.0 (IBM Corp.) and GraphPad Prism 5.0 (GraphPad Software, Inc.) were used for data analysis. Quantitative variables were expressed as mean ± standard deviation or median and interquartile range, which were analyzed using a Student's t-test (for normally distributed variables), Welch's t-test (for data with unequal variance) or one-way ANOVA followed by a Bonferroni post hoc test. Qualitative variables were reported as the number of cases and percentages and compared using a

Table III. Organs infiltrated by myeloid sarcoma.

	Tumor site					
Mouse ID	Uterus	Liver	Skin	Testicle	Pancreas	Ascites
Z-3G49	+					
G3-9-2	+					+
Z-2A105	+	+			+	
Z-3G39	+	+				
Z-2A121					+	
ZNMG3-60			+			
ZNMG3-112		+		+		
ZNMG3-11		+				
ZN-2A52	+					
Z-2A166		+				
ZNMG5-1			+			

χ^2 test or the Fisher's exact test. Kaplan-Meier analysis and the log-rank test were adopted to compare differences in survival time. Survival plots were generated using GraphPad Prism 5.0. P<0.05 was considered to indicated statistically significant differences.

Results

TET2 $^{-/-}$ mice are at high risk of developing MS. All *TET2*$^{-/-}$. mice developed spontaneous lethal hematological malignancies. A total of 2.0% (n=4) of *TET2*$^{-/-}$ mice developed T-cell malignancies, and 5.0% (n=11) developed B-cell malignancies. A total of 93% (n=199) of the *TET2*$^{-/-}$ mice developed myeloid malignancies, which were further classified into MS (n=11, 5.5%) and non-MS (n=188, 94.5%) groups according to the presence of an extramedullary component (Fig. 1A). MS involved multiple extramedullary organs, such as the liver (n=5), uterus (n=5), skin (n=2), testicle (n=1), pancreas (n=2) and ascites (n=1) (Table III), commonly presenting as nodules or organomegaly. Compared with WT mice without any abnormalities, *TET2*$^{-/-}$ mice exhibited a relatively worse outcome, with survival durations ranging from 3 to 25 months, while no significant difference in survival was observed between MS and non-MS mice with *TET2* loss (median survival: MS vs. non-MS=423 days vs. 392 days, respectively, P>0.05; Fig. 1B).

Flow cytometric analyses of MS cells derived from the liver and uterus (ZNMG3-11, ZN-2A52) revealed dominant populations of Mac1$^+$/Gr1$^+$ and high forward scatter myeloid cells (Fig. 1C). Splenomegaly and hepatomegaly were observed in the MS mice (8-15 times and 2-5 times, respectively) compared with non-MS (not WT) mice, respectively (Fig. 1D). The mean weight (g) of the liver in WT, non-MS and MS mice was 1.48, 3.19 and 5.89 g, respectively (P<0.05), and of the spleen 0.26, 0.60 and 2.01 g, respectively (P<0.05) (Fig. 1D). Examination of the hematological parameters revealed significant differences in blood counts among the three groups. The mean WBC count of the WT, non-MS and MS mice was 4.35, 10.94, and 21.36 K/μl, respectively (Fig. 1E, P<0.05), and the mean monocyte (MO) counts were 0.20, 1.41 and 2.82 K/μl, respectively (Fig. 1E, P<0.05). Generally, most MS mice exhibited an increased WBC counts with disproportionate numbers of monocytes (monocytosis), and moderate yet significantly lower red blood cell (RBC) count (M/ul) and hemoglobin level (g/dl) compared with the WT and non-MS mice (Fig. 1E, P<0.01). Among these, 1 MS mouse (Z-2A121) exhibited markedly elevated WBC (119.54 K/μl) and MO (73.39 K/μl) counts. The platelet counts, mean RBC volume and RBC distribution width were comparable among all three mice groups. Consistent with the complete blood counts, morphologic analysis of peripheral blood (PB) smears from the *TET2*$^{-/-}$ MS mice showed dramatically increased leukocytes compared with WT mice, with increased monocytes and/or neutrophils seen in the majority (Fig. 1F). These data suggest that MS mice with *TET2* loss displayed multiple phenotypic characteristics of myeloid malignancies.

Sarcomas in TET2$^{-/-}$ *mice are transplantable*. No recipient receiving BM cells from WT or non-MS tumors developed any evidence of disease within 7 months after transplantation. All the mice that received MS cells developed diseases, became moribund, and had an inferior outcome (Fig. 2A and E). Flow cytometric analysis of the PB cells in the recipients revealed infiltration by a population of uniform, donor cell-derived myeloid malignancy cells (CD45.2$^+$Mac1$^+$/Gr1$^+$), similar to those observed in the donor *TET2*$^{-/-}$ mice (Fig. 2B). Examination of H&E-stained spleen and liver sections from the moribund/deceased *TET2*$^{-/-}$ mice revealed extensive infiltration by intermediate-to-large immature granulocytes with large nuclei and a small amount of cytoplasm, slightly irregular nuclei and dispersed nuclear chromatin. The normal architecture of spleen and liver was effaced and replaced by diffuse atypical granulocyte infiltrates (Fig. 2C). Increased WBC counts with disproportionate numbers of monocytes (monocytosis), and moderate yet significantly lower RBC counts were also detected. No significant difference was found in the PLT counts (Fig. 2D), which resembled those in human myeloid hematological malignancies. The median survival of

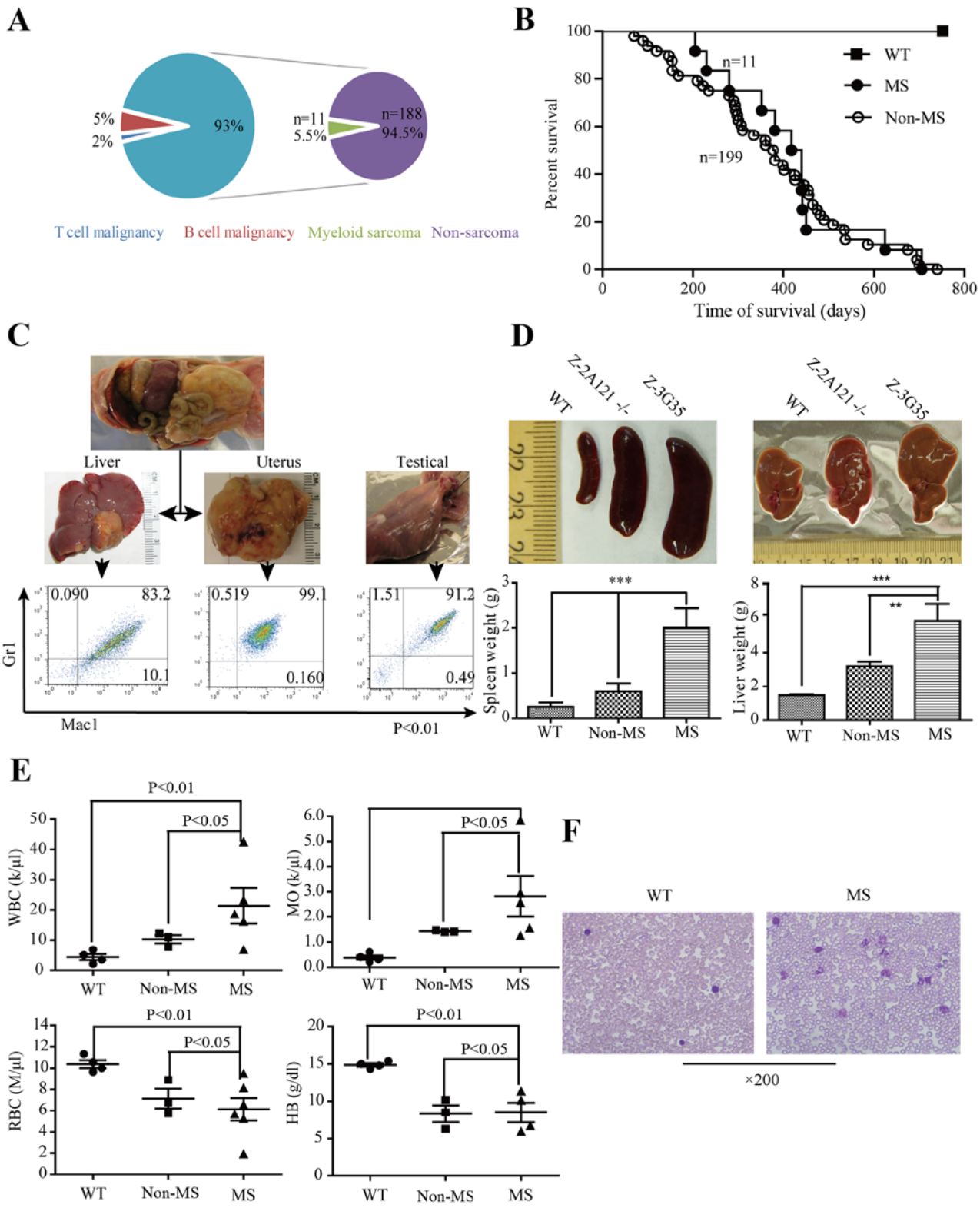

Figure 1. *TET2*$^{-/-}$ mice develop myeloid sarcoma. (A) Proportions of hematological malignancy types developed in 214 *TET2*$^{-/-}$ mice. 214 *TET2*$^{-/-}$ mice developed lethal hematological malignancies, 93% of these *TET2*$^{-/-}$ mice developed myeloid malignancies, of which myeloid sarcomas were observed in 11 (5.5%) mice. (B) Survival of WT (n=67) and *TET2*$^{-/-}$ (n=199) mice with myeloid malignancies over time. Compared with WT mice, *TET2*$^{-/-}$ cohorts with hematological malignancies had a relatively poor outcome, with survival durations ranging between 3 and 25 months. No significant survival difference was found between the sarcoma and non-sarcoma groups. (C) Images of myeloid sarcoma. *TET2*$^{-/-}$ mice developed sarcomas with a phenotype resembling characteristics of myeloid malignancies. Flow cytometric analyses of the liver and uterus (ZNMG3-11, ZN-2A52) cells revealed sarcoma cells with a high forward scatter and mostly positive for Mac1 and Gr1. (D) Spleen and liver weights of non-sarcoma mice (n=4) and sarcoma mice (n=6) as well as age matched WT controls (n=4). These sarcoma mice exhibited striking splenomegaly and moderately enlarged liver compared with non-sarcoma and WT littermates, which were 8-15 times and 2-5 times larger than that of age-matched non-sarcoma mice, respectively. **P<0.01 and ***P<0.001. (E) WT (n=4), non-sarcoma mice (n=4) and sarcoma mice (n=6) were killed and analyzed for PB WBC, MO and RBC counts and Hb levels. Significant differences were identified in WBC counts statistically in comparison between any two group mice. By contrast, the majority of these sarcoma mice exhibited increased WBC counts with disproportionate numbers of monocytes and neutrophils (neutrophilia and monocytosis), moderate yet significantly lower red blood cell counts and hemoglobin levels than WT littermates (P<0.01), but not non-sarcoma mice. (F) Images of May-Giemsa-stained PB smears. Magnification, x200. WT, wild-type; MS, myeloid sarcoma; MO, monocyte.

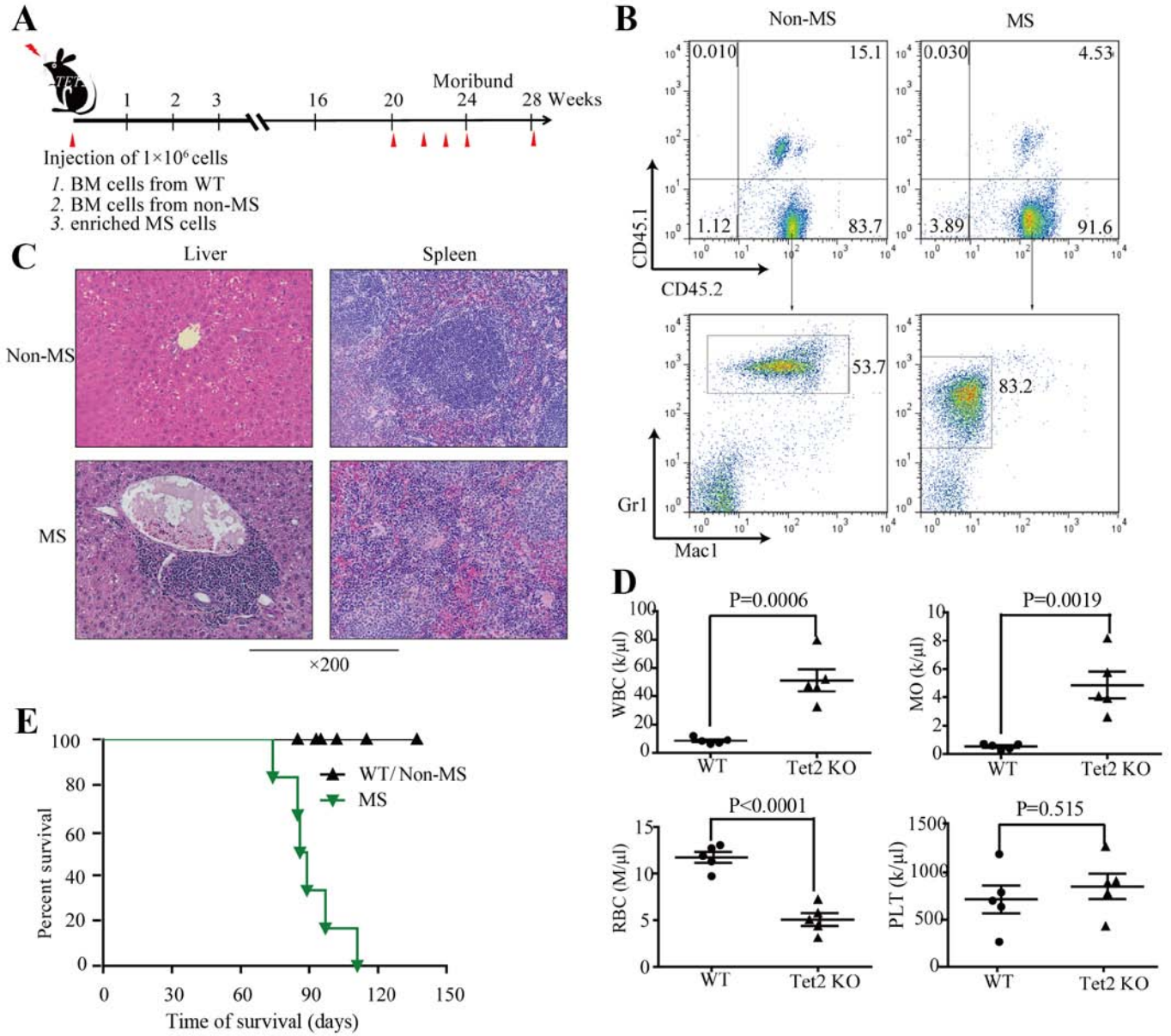

Figure 2. Sarcoma cells in *TET2*$^{-/-}$ mice are transplantable. (A) Tumor transfer schema. Sarcoma cells (1x10^6) from a representative *TET2*$^{-/-}$ mouse with MS or BM cells (1x10^6) from an age-matched WT mouse were injected into sub-lethally irradiated (800 cGy) recipients (n=5). (B) Flow cytometric analyses of peripheral blood myeloid lineage (Mac1+/Gr1+) donor cells (CD45.2+) from a mouse receiving BM cells from a WT mouse or sarcoma cells from a TET2$^{-/-}$ mouse with MS mass. (C) Hematoxylin and eosin-stained histological sections of spleen and liver from a representative recipient mouse. Infiltration of a uniform myeloid malignancy cell population was identified in spleen and liver. Infiltrating patterns and cell morphology were similar to those observed in the donor (MS) mouse. These data demonstrate that the recipients receiving MS cells developed a disease similar to their donor mouse. Magnification, x200. (D) Most of the recipients exhibited elevated WBC monocyte, and decreased RBC counts (n=5). (E) Kaplan-Meier survival curve of sub-lethally irradiated recipients transplanted with BM cells (1x10^6) from a WT mouse or MS cells from *TET2*$^{-/-}$ mice. WT, wild-type; MS, myeloid sarcoma; BM, bone marrow.

the MS recipients was 94.8 days (Fig. 2E). Collectively, these data demonstrated that *TET2*-loss-induced MS was transplantable, suggesting the malignant and neoplastic nature of infiltrated MS cells in *TET2*$^{-/-}$ mice.

Salvage effect of 5-Aza-dC. At the end of the follow-up period, the proportion of mice with myeloid malignancies in the 5-Aza-dC-treated group was markedly lower than that in the control group (6.67 vs. 45.10%, Fig. 3A), with survival durations ranging from 3 to 18.2 months (Fig. 3B). The spleen size of those mice was generally larger compared with that of the WT mice but notably smaller compared with that of the control group. The same trend was observed for the weight of spleens from mice in the three phenotypic groups (Fig. 3C). No deaths occurred in the 5-Aza-dC-treated group. Specifically, one mouse in the control group developed MS in the liver, while no sarcomas were detected in the experimental group. These data indicated the effectiveness of 5-Aza-dC for *TET2*$^{-/-}$ myeloid malignancies, and its potential value in the treatment MS.

Patient characteristics. A total of 436 MDS and 354 AML patients were included in the present study (Tables I and II). MS was found in 38 MDS patients (8.7%) and 32 AML patients (9.0%). A total of 72 MDS patients (16.5%) and 52 AML patients (14.7%) harbored *TET2* deficiency with

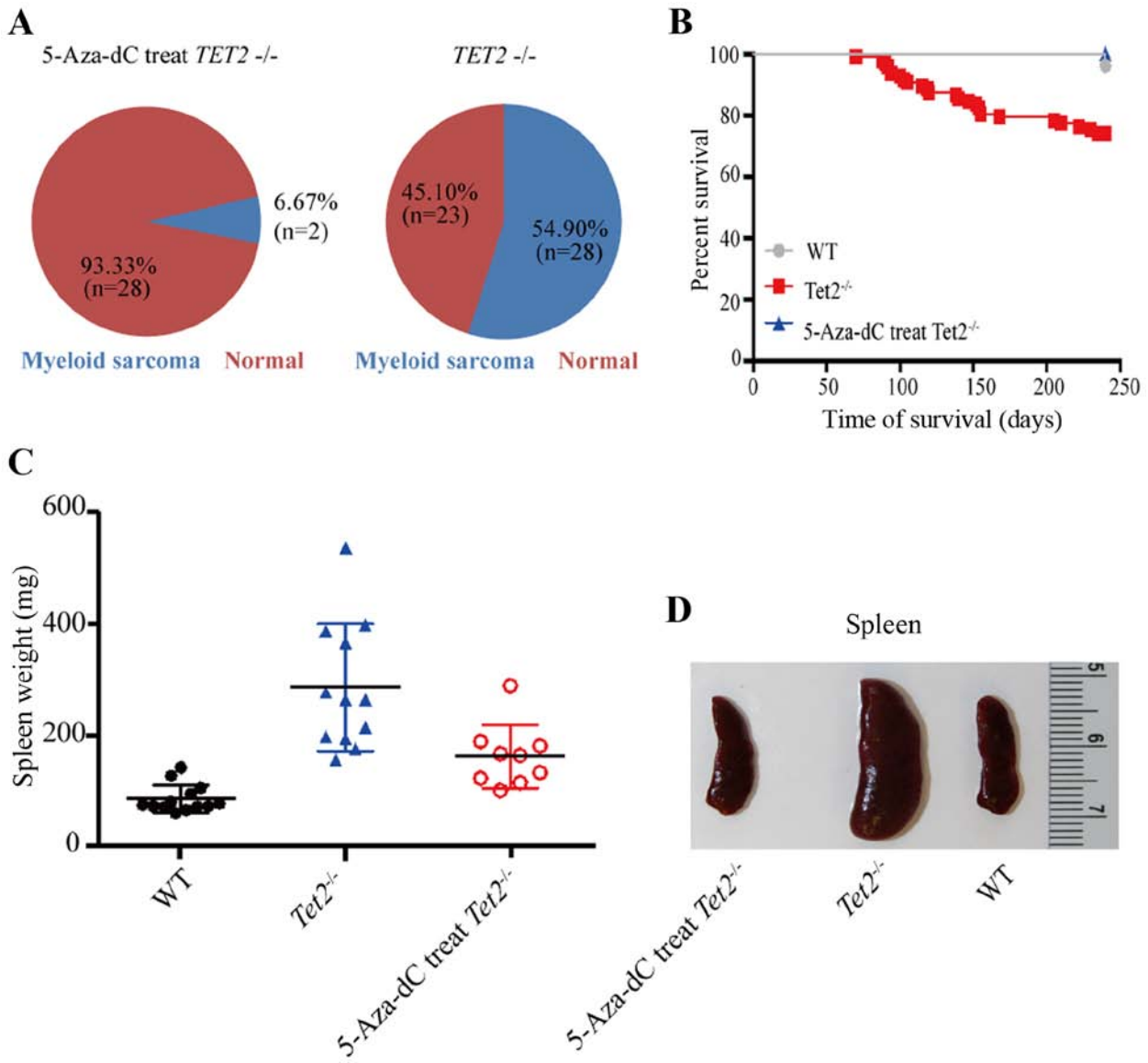

Figure 3. Salvage effect of 5-Aza-dC for *TET2*$^{-/-}$ myeloid malignancies. (A) Proportions of myeloid malignancies in mice of 5-Aza-dC treat *TET2*$^{-/-}$ cohorts were lower after half-a-year of (A) follow-up with (B) an improved survival. (C) Weight of spleen from representative *TET2*$^{-/-}$ mice and *TET2*$^{-/-}$ mice treated with 5-Aza-dC, as well as age-matched WT mice. (D): Photos of spleen from representative *TET2*$^{-/-}$ mice and *TET2*$^{-/-}$ mice with 5-Aza-dC treat, as well as age-matched WT mice. WT, wild-type.

complete information, among whom 8.7% (n=6) of MDS patients and 9.6% (n=5) of AML patients developed MS in the soft tissue (n=6), skin (n=2), oral cavity (n=3) and breast (n=2). The median age of MS patients in MDS and AML was 48 and 41 years, respectively. In MS patients, WBC counts (x10^9) were higher compared with those in patients without MS (MDS, 1.92 vs. 1.27; AML, 1.12 vs. 0.98). However, no significant differences in sex, age, hemoglobin level, or platelet counts were observed between MS and non-MS patients with MDS or AML (P>0.05, Table IV).

Effectiveness of decitabine. Among MDS patients, those with *TET2* mutation had relatively inferior outcomes (Fig. 4). MDS patients with *TET2* deficiency were administered with decitabine monotherapy or combined with CAG (G-CSF for priming, in combination with cytarabine of 10 mg/m^2 q12 h for 14 days and aclarubicin of 20 mg/d for 4 days), and all AML patients received decitabine combined with DA (3 days of daunorubicin and 7 days of cytarabine). Due to the limited-number of MS patients with *TET2* deficiency (Table IV), we were unable to systematically assess the possible impact of *TET2* on survival and further evaluate the efficacy of decitabine with or without chemotherapy for each cohort. An efficacy observation for decitabine was that 3 in 5 MDS patients with MS and 1 in 3 AML patients with MS obtained a partial response (PR), with lesions in the skin and oral cavity decreasing by 25-30%. Hematological improvement was also observed after 2-3 cycles of treatment in patients who were administered decitabine (15 mg/m^2 for 5 days every 28 days). There were no severe adverse events observed in patients administered with decitabine. These results suggest that decitabine monotherapy may be an effective and safe treatment option for MS patients with *TET2* mutations (Table IV).

Table IV. Characteristics of patients with MDS and AML with *TET2* deficiency.

	TET2 deficiency					
	MDS (72,16.5%)			AML (52,14.7%)		
Characteristics	MS(7, 9.7%)	Non-MS	P-value	MS(6, 11.6%)	Non-MS	P-value
Median age, years	43 (16-72)			45 (16-64)		
WBC, x10^9/l	1.92 (0.8-20.53)		0.47[a]	1.12 (0-11.17)		0.13[a]
	1.27 (0-17.32)			0.98 (0.12-10.35)		
Platelets, x10^9/l	128.1 (8-1431)		0.65[a]	102.03 (2-976)		0.82[a]
	134.4 (13-1561)			108.12 (2-1024)		
Hemoglobin	73.65±20.71		0.83[a]	58.78±17.82		0.93[a]
	79.95±24.75			62.46±15.32		
Splenomegaly	2 (33.3%)		0.69[b]	2 (40.0%)		0.73[b]
	24 (36.4%)			21 (44.7%)		
Treatment						
Supportive care		4			2	
AML-induction therapy		11			41	
Decitabine (n=PR, %)	5 (3, 60.0%)			3 (1,33.3%)		0.73[b]
	40 (27,67.5%)			0		
AML-induction therapy + decitabine		9			3	
Cyclosporine		1			3	
HSCT		2			0	

[a]Mann-Whitney U test. [b]Fisher exact test. AML-induction therapy, 3 days of daunorubicin and 7 days of cytarabine. HSCT, hematopoietic stem cell transplant; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome.

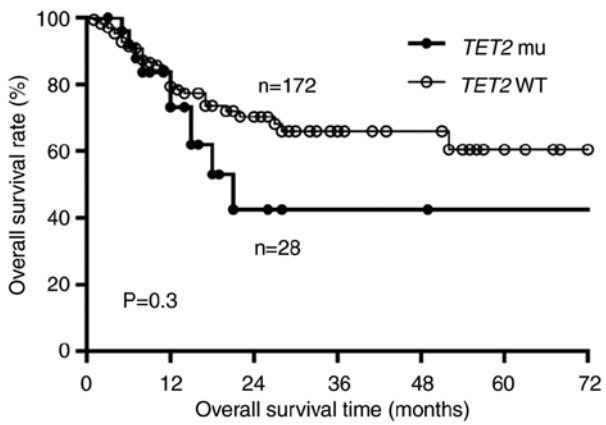

Figure 4. Kaplan-Meier survival curve of patients with myelodysplastic syndrome with *TET2* mutation and WT cohorts. WT, wild-type.

Discussion

In the present study, a *TET2*$^{-/-}$ mouse model was successfully constructed and followed-up. According to the guidelines (25,26), the majority of the mice (93%) developed myeloid malignancies, nearly 95% of which were MDS and AML (27), 5.5% of which (n=11) were accompanied by MS in multiple non-hematopoietic organs. High WBC count, low RBC count and hepatosplenomegaly were distinct characteristics in these MS mice. Histological examination and flow cytometric analysis demonstrated their distinct myeloid characteristics and transplantability, with mice displaying aberrant hematological parameters and Mac1$^+$/Gr1$^+$ and high forward scatter myeloid cells. Kaplan-Meier survival analysis revealed that *TET2* deficiency may be an negative factor in hematological malignancies, but did not adversely affect the OS of the MS mice. As an important demethylation enzyme, *TET2* deficiency often leads to DNA hypermethylation. Therefore, the effect of HMAs on MS mice and patients with *TET2* mutations was examined. The animal experiments demonstrated that *TET2*-deficient MS mice exhibited typical myeloid characteristics but not inferior survival, which indicated that *TET2* mutations may not be an independent prognostic factor and may play a role with other possible co-mutations, which warrants further investigation. As expected, 5-Aza-dC was successful in reducing the incidence of MS with *TET2* mutation.

In the present study, the incidence of MS with *TET2* deficiency was 11.6 and 9.7% for AML (n=6/52) and MDS patients (7/72), respectively, which is similar to the results of our animal experiment but higher than the 2.5-9.1% of patients with AML in other reports (2-5,14). This discrepancy may be attributed to our limited sample, the bias of disease subtype and ethnic differences. In the present study, the median age of these MS patients was 44 years old in this study. In addition, AML and MDS patients with MS in this study displayed high leukocyte counts, as expected.

Due to the performance status, medical comorbidities, toxicity and uncertain benefit of standard induction chemotherapy in older patients (more than 50 years) with myeloid

malignancies, conventional AML induction therapy (3 days of daunorubicin and 7 days of cytarabine) is poorly tolerated and increases the mortality rate. Thus, treatment is more challenging in elderly patients. In recent years, HMAs have been reported to alter methylation status in some patients with MS and decrease the tumor mass with acceptable toxicity (19,28,29). In the present study, 3/5 MDS patients had a response to decitabine, and there was 1/3 ORR in AML patients, similar to the characteristics displayed by the mice, with skin and oral cavity lesions decreasing by 25-30% and hematological improvement without a serious toxicity profile. Hematologic improvement was also observed after 2-3 cycles of treatment in patients who were administered decitabine (15 mg/m^2 for 5 days every 28 days). There were no reported severe advent events. However, due to the low incidence of MS and the drug accessibility, we were unable to recruit a sufficient number of patients to comprehensively evaluate the characteristics of MS and the effect of HMAs. We were also unable to group patients for further study to evaluate the effect of decitabine. Since 5-AZA is not available in China until 2018, we administrated different drugs for mice and patients here which was a limitation for our study. To summarize, it was deduced that decitabine may be an effective and safe treatment option for MS patients with *TET2* mutations. Based on the results of the animal experiments and patient validation, it was concluded that *TET2* deficiency plays a role in MS and its prognostic significance requires further investigation. HMAs may be a beneficial treatment for such patients.

MS is a complex disease. A previous report proposed that specific cytogenetic abnormalities in MS may predict the site of involvement (30). Hence, the identification of multiple factors associated with MS and their underlying mechanisms is particularly important. A larger sample size and more accurate studies are required to identify and interpret these differences and their implications, providing new insights into a potential target for therapeutic intervention in MS. To the best of our knowledge, the association of *TET2* abnormalities with MS has not been previously explored. Importantly, the *TET2*$^{-/-}$ mice used in the present study may serve as a model to investigate the association between *TET2* loss and diverse hematological malignancies (including MS), and to explore possible treatment regimens for patients.

In conclusion, *TET2* deficiency appears to have an impact on MS and its prognostic significance must be further investigated, as it may be a potential treatment biomarker for MS patients. This finding stresses the scarcity of studies in this field and more studies are urgently needed to address crucial questions on methylation and demethylation as a target for MS therapy. A larger data registry and more controlled clinical correlative studies are required to more accurately assess the effect of *TET2* deficiency on the prognosis, diagnosis, and therapeutic relevance of MS and the role of HMAs in the treatment of MS.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JW, ZM and YJ performed the experiments. PZ, WL, XT, SC, FY, MX and HW provided material, patients' follow-up and conducted initial analysis of the data in their center. YL and DX were responsible for performing the FACS experiments and generating FACS images. JW and ZM participated in interpretation of data. JW, ZM and YJ wrote the paper. ZC, HW and ZZ designed and supervised the research. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Second Hospital of Tianjin Medical University. Experiments with animals were performed in accordance to the guidelines for experimental animal management established by Tianjin Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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