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Aberrant alternative splicing of human zinc finger gene ZNF268 in human hematological malignancy

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Abstract. The human ZNF268 gene was initially described as a gene associated with early human embryogenesis and was later implicated in human leukemia due to the identification of an alternatively splice form in leukemia patients. To systematically evaluate the correlation of ZNF268 with human hematological malignancy, expression of different alternatively spliced forms of ZNF268 mRNA in peripheral blood of 45 patients with hematological malignancies and 17 healthy donors were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and nested PCR. We demonstrated that presence of ZNF268a, ZNF268c, ZNF268f and ZNF268g were significantly different between the patients and healthy donors (P<0.05). Our study thus suggests that aberrant alternative splicing of ZNF268 is a potential prognostic factor of and may contribute to human hematological malignancies.

Introduction

Hematological malignancies, arising from blood forming cells and from cells of the immune system, account for ~8% of malignancies in humans. There are many causes for the genesis of hematological malignancies including radiation, chemicals, viruses and genetic factors, all of which contribute to the diseases via disturbing the regulation of hematogenesis to a large degree. Transcription factors play a major role in the differentiation of various hematopoietic lineages (1-4). Zinc finger genes comprise the largest family of transcription factors in vertebrate organisms. During the past decades, many human zinc finger genes have been proven to be associated with hematological malignancies, such as Egr-1 (5), PLZF (6) GATA1 (7) and WT1 (8).

Human zinc finger gene ZNF268, cloned and characterized from a cDNA library of 3-5-week-old human fetus in our laboratory (9), is a typical KRAB-containing zinc finger gene. Krackhardt and colleagues identified Kw-4 by SEREX, one of the alternative transcripts of ZNF268, as one of tumor-associated antigens in chronic lymphocytic leukemia (10). Functional research into the promoter of ZNF268 demonstrated that ZNF268 was directly regulated by CREB (11), which was proposed as a proto-oncogene in hematopoiesis and in acute myeloid leukemia (12). A transcriptional profiling of human hematopoiesis during *in vitro* lineage-specific differentiation indicate the expression of ZNF268 continuously decreased during erythropoietic differentiation (13). The above collectively suggest that ZNF268 may play a role in the differentiation of various hematopoietic lineages and in the genesis of hematological malignancies. In addition, multiple splice variants, including ZNF268a, ZNF268b, ZNF268c, ZNF268d, ZNF268e, ZNF268f and ZNF268g, were identified in several human tumor-derived cell lines, human fetal tissues (14) and peripheral blood from healthy adults (15). It has been suggested that more than half of human genes are subject to alternative splicing events and sometimes aberrant splice variations cause for genetic diseases (16,17). Therefore, detecting these events of a human gene is vital to biomarker discovery across a variety of human diseases (18,19). Thus, given the possible involvement of ZNF268 in leukemia, we were interested to know whether the differential expression of alternative splicing events in ZNF268 occurs in patients with hematological malignancies.

In this study, we investigated the expression of different splice variants in the peripheral blood of 45 patients with hematological malignancies and 17 healthy donors by RT-PCR and nested PCR. Further statistic analysis showed significantly differential expression of ZNF268a, ZNF268b, ZNF268c, ZNF268f and ZNF268g in certain hematological malignancies compared to healthy donors (P<0.05), suggesting that alternative splicing in ZNF268 may serve as a biomarker for hematological malignancies and that ZNF268 may play a role in such diseases.

Materials and methods

Patients. Of each heparinized peripheral blood (PB) sample from 45 patients with hematological malignancies (23 AML,

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Abbreviations: AML, acute myeloid leukemia; ALL, acute lymphoid leukemia; CML, chronic myeloid leukemia; NHL, non-Hodgkin lymphoma

Key words: ZNF268, aberrant alternative splicing, hematological malignancy

Table I. Clinical characteristics of patients.

Samples	No. of patients	Age (median)
AML	23	14-74 (40)
ALL	7	15-41 (21)
CML	7	15-40 (29)
CLL	3	68-72 (69)
Hypoplastic leukemia	1	43
Malignant histocytosis	1	8
NHL	3	22-63 (59)
Total	45	8-74 (37)

Table II. Primers used in this study.

Primer name	Primer sequence
BU1	5'-GTTGCGAACCCTTCTGGC-3'
BD1	5'-TCCATGTACCTGAAACCCATTAG-3'
BUN1	5'-ATCTGGAGGTGGAGGCAGTA-3'
BDN2	5'-ATGTACCTGAAACCCATTAGGAT-3'
BDN3	5'-CAGCAACAAGGCGTCATC-3'
e3s	5'-TCCGGATCCGAAACAGTTCATGGG-3'
e5a	5'-GGACAGGTCTGATTTGGAACTTGGG-3'
e4s	5'-GTAATTCATGGATGTGTTTGTGG-3'
ß-actin 1	5'-GCTCGTCGTCGACAACGGCTC-3'
ß-actin 2	5'-CAAACATGATCTGGGTCATCTTCTC-3'

7 ALL, 7 CML, 3 CLL, 1 hypoplastic leukemia, 1 malignant histocytosis, 3 NHL) 1 ml was taken at the time of their initial diagnosis after informed consent. The clinical features are listed in Table I. Seventeen samples of PB were obtained as the control from healthy donors after informed consent. The blood samples were obtained from local hospitals.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA of each sample was extracted using TRIzol LS™ (Invitrogen, Life Technologies, Grand Island, NY) according to the manufacturer's protocol. To remove genomic DNA contamination, DNase treatment was performed as recommended in the manufacturer's kit. First-strand cDNA was made using RNA PCR kit (Takara, Japan) with oligo dT-Adaptor primer in a 10 μl reaction volume containing 500 ng of total RNA. One-tenth volume of the RT product was amplified using EX Taqase PCR system (Takara) with the primers BU1 and BD1 (Table II) or with the primers ß-actin 1 and ß-actin 2 (Table II) in a total volume 25 μl. For amplification of ZNF268, the samples underwent 30 cycles of 94˚C for 45 sec, 58˚C for 45 sec and 72˚C for 60 sec. For the amplification of ß-actin, the samples underwent 20 cycles of 94˚C for 45 sec, 55˚C for 45 sec and 72˚C for 30 sec. The PCR products were separated on 1.5% agarose gels and the results was recorded by Kodak electrophoresis documentation and analysis system 290 (EDAS 290).

Nested PCR and sequencing analysis. Dilution (1:100) of the PCR products for ZNF268 were used for the second-round amplifications using the primer pairs of BUN1 and BDN2, BUN1 and BDN3, e4s and BDN2 and e3s and e5a, respectively (Table II). PCR was performed in standard Biostar™ Taq polymerase system (Biostar International Co., Canada) and consisted of an initial denaturation step at 94˚C for 5 min, followed by 30 cycles of 94˚C, 45 sec; 55˚C, 45 sec; 72˚C, 60 sec and a final extension step of 7 min at 72˚C. The PCR products were separated on 1.5% agarose gels and the results was recorded by Kodak EDAS 290. The products of nested PCR with primers BUN1 and BDN2 were purified by E.Z.N.A.™ Gel Extract kit (Omega, USA), ligated into pGEM-T Easy Vector (Promega Corporation, USA) and then transformed into *Escherichia coli*. For each transformation of each kind of PCR product, 3 colonies were randomly picked and the inserts were determined by PCR. Representative clones were sequenced and sequencing results were analyzed with nucleotide blast in GenBank of NCBI (www.ncbi.nlm.nih.gov).

Statistical analysis. The 45 patients were divided into three groups according to gender, age and diagnosis subtype. By Chi-square tests the frequencies for the presence of the splice variants were analyzed between or among the samples in each group. Fisher's exact tests were performed by online tool 'Consultancy for Research and Statistics' (http://home.clara.net/sisa/fisher.htm) to compare the frequencies for the presence of the splice variants between a certain subtype of patients and healthy donors.

Results

Identifying splice variants of ZNF268 in patients and healthy donors. Previous studies showed that ZNF268 gene had multiple splice variants in human tumor-derived cell lines and fetal tissues (14) and in peripheral blood from healthy adults (15). In order to determine possible differential distribution of those splice variants in patients with hematological malignancies and healthy donors, we analyzed ZNF268 and ß-actin in the peripheral blood of 45 patients with various hematological malignancies and 17 healthy donors by RT-PCR. While the ß-actin analysis worked well as expected, the signals for ZNF268 were barely detectable (data not shown). To improve the sensitivity in detecting ZNF268, we performed nested PCR with the primers BUN1 and BDN2 (Table II). The predicted lengths of these PCR products were 886, 780, 685, 653, 558, 557, 452 and 356 bp for ZNF268b, ZNF268a, ZNF268g, ZNF268c, ZNF268f, ZNF268e, ZNF268d and Kw-4, respectively. As shown in Fig. 2A, 7 distinct bands were observed when the PCR products were separated on an agarose gel. Cloning and sequencing of these distinct products revealed 8 distinct mRNA species. Sequence alignments with ESTs in GenBank of NCBI (www.ncbi.nlm.nih.gov) showed that they were all derived from different splice variants: ZNF268a (Genbank accession no.: AF317549), ZNF268b (AF385187), ZNF268c (DQ057356), ZNF268d (DQ057357), ZNF268e (DQ057358), ZNF268f (DQ057359), ZNF268g (DQ057360) and Kw-4 (AF432217) (Fig. 1A).

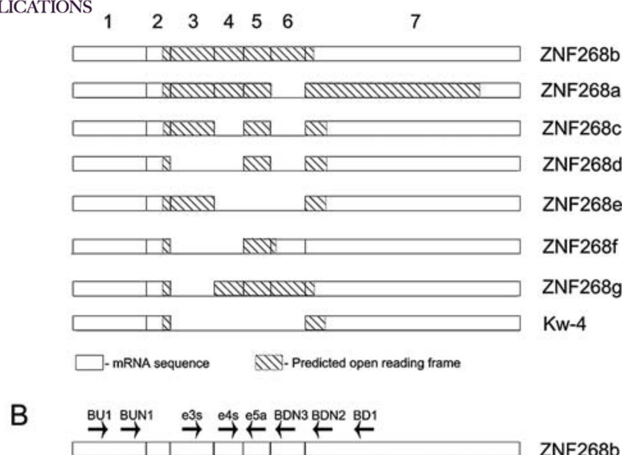


Figure 1. (A) Schematic presentation of the alternative splicing forms of ZNF268 and their predicted open reading frames. (B) Locations of the primers used for the RT-PCR analysis.

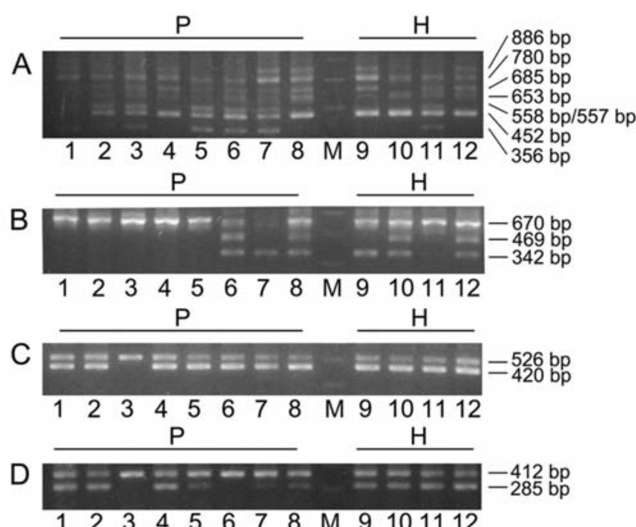


Figure 2. Representative nested RT-PCR analysis of the expression of ZNF268 splice variants. (A) PCR products using primers BUN1 and BDN2. Lanes 1-8, 8 different patients and lanes 9-12, 4 healthy donors. Bands correspond to following products: 886 bp-ZNF268b, 780 bp-ZNF268a, 685 bp-ZNF268g, 653 bp-ZNF268c, 558 bp/557 bp-ZNF268f/ZNF268e, 452 bp-ZNF268d, 356 bp-Kw-4. (B) PCR products using primers BUN1 and BDN3. Lanes 1-8, 8 different patients and lanes 9-12, 4 healthy donors. Bands correspond to following products: 670 bp-ZNF268b, 469 bp-ZNF268g, 342 bp-ZNF268f. (C) PCR products using primers e4s and BDN2. Lanes 1-8, 8 different patients and lanes 9-12, 4 healthy donors. Bands correspond to following products: 526 bp-ZNF268b and/or ZNF268g, 420 bp-ZNF268a. (D) PCR products using primers e3s and e5a. Lanes 1-8, 8 different patients and lanes 9-12, 4 healthy donors. Bands correspond to following products: 412 bp-ZNF268b and/or ZNF268a, 285 bp-ZNF268c. P, patient; H, healthy donor and M, GeneRuler™ 1 kb DNA Ladder (MBI Fermentas).

Further detection of splice variants in patients and healthy donors. As shown in Fig. 1A, among the 8 splice forms of ZNF268 identified so far, only ZNF268b contains all 7 exons. Absence of exons happens in exons 3, 4, 5 and 6 while exons 1, 2 and 7 are present in all variants. Thus, all 8 splice variants could be detected by using primers BUN1 (located in exon 1, Fig. 1B) and BDN2 (located in exon 7, Fig. 1B). However, not all the splice variants could be effectively amplified

Table III. Clinical characteristics of the patients and relationship to the expression of splice variants ZNF268a, ZNF268b, ZNF268c, ZNF268f and ZNF268g.

	n	ZNF268a		P[c]	ZNF268b		P[c]	ZNF268c		P[c]	ZNF268f		P[c]	ZNF268g		P[c]
		+[a]	-[b]		+[a]	-[b]		+[a]	-[b]		+[a]	-[b]		+[a]	-[b]	
Total	45	38	7		45	0		28	17		25	20		18	27	
Gender				NS			NS			NS			NS			NS
Male	28	21	7		28	0		18	10		16	12		11	17	
Female	17	17	0		17	0		10	7		9	8		7	10	
Age (years)				NS			NS			NS			NS			NS
0-14	3	3	0		3	0		2	1		2	1		0	3	
15-60	36	29	7		36	0		21	15		19	17		14	22	
>60	6	6	0		6	0		5	1		4	2		4	2	
Diagnosis				NS			NS			NS			NS			NS
AML	23	21	2		23	0		15	8		15	8		9	14	
ALL	7	4	3		7	0		5	2		4	3		4	3	
CML	7	6	1		7	0		3	4		3	4		2	5	
CLL	3	3	0		3	0		3	0		2	1		3	0	
NHL	3	3	0		3	0		1	2		0	3		0	3	
Hypoplastic Leukemia	1	0	1		1	0		0	1		0	1		0	1	
Malignant Histocytosis	1	1	0		1	0		1	0		1	0		0	1	

[a]Presence of the splice variants as determined by nested PCR detection. [b]Absence of the splice variants as determined by nested PCR detection. [c]P-value (Chi-square with Yates' correction when appropriate). NS, not significant (P>0.05).

Table IV. Statistical analysis of ZNF268a, ZNF268b, ZNF268c, ZNF268f and ZNF268g in patients vs. healthy donors.

	ZNF268a		ZNF268b		ZNF268c		ZNF268f		ZNF268g	
	Positive [a]/total	Frequency of presence %	Positive [a]/total	Frequency of presence %	Positive [a]/total	Frequency of presence %	Positive [a]/total	Frequency of presence %	Positive [a]/total	Frequency of presence %
Healthy donors	17/17	100	17/17	100	16/17	94.1	14/17	82.4	4/17	23.5
Patients[b]	38/45	84.4	45/45	100	28/45[c]	55.6	25/45[c]	55.6	18/45	40
AML	21/23	91.3	23/23	100	15/23[c]	65.2	15/23	65.2	9/23	39.1
ALL	4/7[c]	57.1	7/7	100	5/7	71.4	4/7	57.1	4/7	57.1
CML	6/7	85.7	7/7	100	3/7[c]	42.9	3/7	42.9	2/7	28.6
CLL	3/3	100	3/3	100	3/3	100	2/3	66.7	3/3[c]	100
NHL	3/3	100	3/3	100	1/3[c]	33.3	0/3[c]	0	0/3	0

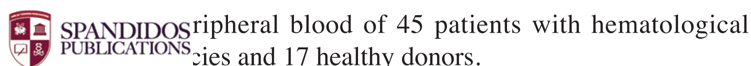
[a]Presence of splice variants ZNF268a, ZNF268b, ZNF268c, ZNF268f and ZNF268g as determined by successful detection by nested PCR respectively. [b]Forty-five patients including 23 AML, 7 ALL, 7 CML, 3 CLL, 3 NHL, 1 hypoplastic leukemia and 1 malignant histocytosis, among which the 23 AML, 7 ALL, 7 CML, 3 CLL and 3 NHL was analyzed respectively below. [c]P<0.05 according to exact Fisher's test.

because of competition in a PCR reaction. For further detection of these splicing forms, we designed additional primers. Exon 6 is present in ZNF268b, ZNF268g and ZNF268f but not in ZNF268a, ZNF268c, ZNF268d, ZNF268e and Kw-4. So with primers BUN1 (located in exon 1, Fig. 1B) and BDN3 (located in exon 6, Fig. 1B), only ZNF268b, ZNF268g and ZNF268f were amplified while ZNF268a, ZNF268c, ZNF268d, ZNF268e and Kw-4 were not (Fig. 2B). By utilizing the same strategy, ZNF268a was distinguished from ZNF268b and/or ZNF268g with primers e4s and BDN2 (located in exons 4 and 7, respectively, Fig. 1B) (Fig. 2C); ZNF268c was well separated from ZNF268b and/or ZNF268a by using primer pair of e3s and e5a (located in exons 3 and 5, respectively, Fig. 1B) (Fig. 2D). Using these different primer sets, we showed that ZNF268b was present in all of 12 representative samples in Fig. 2B, including 8 patients and 4 healthy donors, while ZNF268g was absent in 6 of 8 patients (shown in lanes 1, 2, 3, 4, 5 and 7, Fig. 2B) and in 2 of 4 healthy donors (shown in lanes 9 and 11, Fig. 2B). ZNF268f was absent in 5 of 8 patients (shown in lanes 1, 2, 3, 4 and 5, Fig. 2B) and in 1 of 4 healthy donors (shown in lane 11, Fig. 2B). Fig. 2C showed that ZNF268a was absent in 1 of 8 patients (lane 3) and in none of 4 healthy donors. ZNF268c was absent in 2 of 8 patients (Fig. 2D, lanes 3 and 6) and in none of 4 healthy donors. We then analyzed the frequencies for the presence of ZNF268a, ZNF268b, ZNF268c, ZNF268f and ZNF268g by Chi-square test. The statistical analysis showed that the expression of ZNF268a, ZNF268b, ZNF268c, ZNF268f and ZNF268g had no correlations to gender, age and diagnosis subtypes of the patients (Table III).

Differential distributions of the splice variants between the patients and healthy donors. We compared the frequencies for the presence of ZNF268a, ZNF268b, ZNF268c, ZNF268f and ZNF268g respectively between the patients and the healthy donors using Fishers' exact tests. The frequencies for the presence of ZNF268c and ZNF268f were markedly lower in 45 patients (P<0.05, Table IV). Furthermore, the frequency for the presence of ZNF268a was also much lower in patients with ALL (P<0.05, Table IV). The frequency for the presence of ZNF268c was much lower in patients with AML, CML and NHL (P<0.05, Table IV). For ZNF268f, it was absent in all 3 patients with NHL, compared to in 14 of the 17 healthy donors (P<0.05, Table IV). Interestingly, ZNF268g was present in all 3 patients with CLL, compared to in 4 of the 17 healthy donors (P<0.05, Table IV). On the other hand, ZNF268b was present in all the patients and healthy donors.

Discussion

Alternative splicing is a critical mode of genetic regulation in higher eukaryotes (20). It is now well established that most human genes express more than one mRNA by alternative splicing (21). Moreover, many gene transcripts have multiple splicing patterns and some have thousands (22,23). It was reported that ZNF268 was expressed in several human fetal tissues, cell lines (14) and peripheral blood from healthy adults (15) in the terms of multiple splicing forms. In this study, we showed that some forms were differentially present



ripheral blood of 45 patients with hematological cies and 17 healthy donors.

Cancer is considered mostly as a genetic disease. Thus, it is not surprising that aberrant splicing variation caused by infidelities in the process of alternative splicing is often associated with cancer and aberrations in the splicing pattern are commonly seen in many cancer-related genes (19,24). For example, splicing alterations in 63% of sporadic Wilm's tumors implicated a role of Wilm's tumor (25). Similarly, different FHIT transcripts via alternative splicing in anal cancers and in normal samples were heterogeneous, suggesting a relationship of this gene with anal cancer (26). Additionally, three new splicing variants restricted to pre-B and C-ALL indicated that p73 participated in B cell ALL differentiation (27). Splicing variants of IFR-1 were differentially expressed in MDS patients, suggesting that the alternative splicing of IFR-1 might be used as a diagnostic factor for the disease (28).

Here, for the first time, we showed that ZNF268c and ZNF268f were absent preferentially in patients with hematological malignancies compared to healthy donors ($P<0.05$, Table IV). In addition, while ZNF268a and ZNF268b were both present in all healthy donors, the absence of ZNF268a in patients was significant compared to health adults (Table IV). On the other hand, ZNF268g was not present similarly in the patients and healthy donors (Table IV). Interestingly, further analysis within different subtypes of the disease revealed that ZNF268g was expressed in all 3 patients with CLL (100%) compared to 4 of 17 healthy donors (23.5%) ($P<0.05$, Table IV) while ZNF268a, ZNF268c and ZNF268f showed lower frequencies in certain subtypes of the patients ($P<0.05$, Table IV). These findings suggest that the alternative splice variants of ZNF268 might be prognostic factors or potential biomarkers for hematological malignancies.

It is now apparent that variability in splicing patterns is a major source of protein diversity from the genome (20). However, the prevalence of alternative splicing and our limited understanding of its mechanisms present a challenge for identifying all the proteins available to an organism during normal and pathological processes (19). Among the 8 splicing variants of ZNF268, ZNF268a has been demonstrated to have a corresponding protein product, a full length KRAB-containing zinc finger protein (~108 kDa), which can enter into cell nucleus and have transcription activation property (14). ZNF268c, ZNF268f and ZNF268g have open reading frames shorter than that of ZNF268a (Fig. 1A). The molecular weights of their predicted proteins are 19.5, 5.1 and 14.2 kDa, respectively. However, the corresponding proteins of the three splicing forms have not been reported so far. Considering that ZNF268c, ZNF268f and ZNF268g share the same KOZAK ATG with ZNF268a (Fig. 1A), we expect that corresponding shorter proteins will be made *in vivo*. Indeed, when their coding regions were inserted into pEGFP-N1 and expressed in HEK293T, we were able to detect the expected size EGFP-fusion proteins with a specific anti-EGFP antibody (data not shown). As ZNF268a, ZNF268c, ZNF268f and ZNF268g were aberrantly expressed in hematological malignancies, they might participate in the genesis of hematological malignancies via the corresponding protein products.

In conclusion, our results showed that the aberrant splice variation of ZNF268 was correlated with hematological malignancies. Additionally, ZNF268c, ZNF268f and ZNF268g may encode the corresponding proteins. Therefore, further studies on detecting the expression of ZNF268 both at RNA and protein level in larger numbers of patients would add value to clinical diagnosis of hematological malignancies.

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