Copy Number Variations of EphA3 Are Associated With Multiple Types of Hematologic Malignancies

Ming Guan,[1a] Li Liu,[2a] Xiaosu Zhao,[3] Qi Wu,[4] Bo Yu,[4] Yong Shao,[4] Hong Yang,[5] Xinrong Fu,[6] Jun Wan,[4,6b] Wei Zhang[7b]

Abstract

Background: EphA3 is a component of the Eph receptor family, the largest subgroup of the receptor tyrosine kinase (RTK) family. A recent array-based study implicated the presence of copy-number variations (CNVs) of EphA3 in the genomes of acute myelogenous leukemia. CNVs are present in the general population at varying degrees, and have been found to associate with various types of diseases including hematologic malignancies. However, most of the current studies focused on the genome-wide screening of CNVs, and the functional impact of such regions needs to be extensively investigated in large number of clinical samples. Patients and Methods: In our study, we collected 617 bone marrow samples from multiple types of hematologic malignancies as well as healthy controls. DNA copy numbers and mRNA levels of EphA3 in these samples were examined. Results: We found significant association between the CNVs of EphA3 and these hematologic malignancies including acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), multiple myeloma (MM), and myelodysplastic syndrome (MDS). We also observed a positive correlation between the relative mRNA level and gene dosage of EphA3. Conclusion: The CNVs of EphA3 were associated with multiple types of hematologic malignancies including ALL, AML, CLL, CML, MM, and MDS.

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Introduction

EphA3 is a component of the Eph receptor family, the largest subgroup of the receptor tyrosine kinase (RTK) family. It is divided into 2 subclasses, A and B, based on distinct structural properties of their ligands, the ephrins.[1] The Eph receptors and ephrins are membrane-bound proteins whose interactions initiate unique bidirectional signaling events.[2] This receptor/ligand system is associated with various signaling pathways related to cell growth and viability, cytoskeletal organization, cell migration, and antiapoptosis. Altered expression of Ephs and ephrins is associated with angiogenesis and tumor vasculature in many types of human cancers, including breast, lung, prostate cancers, melanoma, and leukemia.[2-4] EphA3 expression is known to be associated with B- and T-cell malignancies.[5-7] The expression of EphA3 was reported to be induced by IGF-I in neoplastic but not in normal T cells.[8] The EphA3 receptor is increased without apparent amplification or rearrangement in human lymphoid tumor cell lines, which raises the possibility that EphA3 is a contributing factor in lymphoid malignancy.[6] In non-lymphocytic tumor cells, EphA3 was detected in a subset of rhabdomyosarcoma (RMS) cell lines and suppressed cell adhesion and migration.[9] Genetic alterations of the EphA3 gene were detected in hepatocellular carcinoma by single-strand conformational polymorphism and sequencing.[10] And the recent array-based CNV study further linked EphA3 to hematologic malignancies.[11]

Copy-number variations (CNVs) were originally defined by the presence of variable numbers of copies of large, multikilobase

[1]Department of Clinical Laboratory, Huashan Hospital, Shanghai, China
[2]Department of Anatomy, Capital Medical University, Peking, China
[3]Department of Hematology, Peking University People's Hospital, Peking University Institute of Hematology, Beijing, China
[4]Biomedical Research Institute, Shenzhen-PKU-HKUST Medical Center, Shenzhen, Guangdong, China
[5]Department of Clinical Laboratory, Shenzhen Hospital Peking University. Shenzhen, Guangdong, China
[6]Department of Life Science, the Hong Kong University of Science and Technology, Hong Kong, China
[7]JNU-HKUST joint lab, Ji-Nan University, Guangdong, China
[a]These authors contributed equally to this work.
[b]These authors contributed equally to this work.

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Address for correspondence: Wei Zhang, PhD, JNU-HKUST joint lab, Ji-Nan University, Guangdong province, China. 510632
Fax: 86-20-852-22-120; e-mail: zhangwei@ust.hk

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genomic regions in the genomes of different individuals.[12-20] However, recent high-resolution genome maps have revealed smaller CNVs among healthy humans,[15,21] thus extending the definition of CNVs to the length of regions being as short as several hundred bases. CNVs are present in the general population at varying degrees, and their role in disease is poorly defined so far. Most CNVs have either no phenotypic consequences or only subtle or benign ones. Several methodologies, such as the most commonly used array-based comparative genomic hybridization (aCGH), were used for genome-wide CNV detection and genotyping. CNVs have been discovered to have phenotypic consequences and associate with various types of diseases including mental disorders, rheumatoid arthritis, diabetes, and so on.[22-25] The implication of CNVs in cancers has become a hot spot over the past few years.[26-28] Studies using SNP arrays and aCGH have suggested that CNVs are common in genomes of hematologic malignancies.[29-33] Hematologic malignancies are the types of cancer that affect blood, bone marrow, and lymph nodes. Major types of hematologic malignancies include leukemia, lymphoma, and myeloma. A recent study screened paired tumor and normal samples to identify genes that are somatically altered in leukemia genomes, and implicated that EphA3 was deleted in tumor samples.[11]

However, most of the aCGH experiments focused on the genome-wide screening of CNVs, and the data obtained are generally informative but not definitive. Thus, they require further molecular genetic experiments for validation. The functional effect of such regions, especially the particular genes, warrants extensive studies. And the significance of these identified CNVs needs to be confirmed in large number of clinical samples. So, in our study, we collected 617 bone marrow samples from multiple types of hematologic malignancies including acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), multiple myeloma (MM), myelodysplastic syndrome (MDS), and healthy controls. We aim to extensively examine the significance of CNVs of EphA3 in these hematologic malignancies.

Materials and Methods

Controls and Patient Samples

Aspirated bone marrow samples from 114 AML, 86 ALL, 78 CML, 72 CLL, 67 MM, 98 MDS, and 102 healthy controls were collected at Peking University People's Hospital. The median age of all patients was 42 years, ranging from 11 to 79 years. All patients were of Chinese ancestry. All the patients were untreated, and all the bone marrow samples were collected at the time of diagnosis. All the samples (except for normal controls) were characterized to be positive for neoplastic infiltrate by examination of bone marrow smear. Definition and classification of hematologic malignancies were based on 2008 World Health Organization (WHO) Classification of Tumors of Hematopoietic and Lymphoid Tissues.[34] The study was approved by the ethical committee of Peking University Shenzhen Hospital. The individuals gave their written informed consent. The investigations were conducted according to the Declaration of Helsinki principles.

DNA Extraction and Quantification of Copy Numbers

Genomic DNA was isolated from the tissues using the Genomic DNA Extraction Kit (Innogent, Shenzhen, China) according to the manufacturer's instruction. Quantitative PCR was performed through BioRad Chromo4 real-time PCR system. Average copy numbers of RNAse P in normal candidates (copy numbers = 2) were used as control.[18] The copy numbers of EphA3 was calculated by using the comparative C(T) method. Cut-off values of 0.25, 0.75, 1.25, and 1.75 were used to define the copy numbers as 0, 1, 2, and 3, respectively. The primers for RNAse P are: forward: 5' AGACTAGGGTCAGAAGCAA and reverse: 5' CATTTCACTGAATCCGTTC. The primers for EphA3 are: forward: 5' GTTGCCTTGGTGTCTGTG and reverse: 5' AAGCCATTCGCCTTCTGT. Statistical analysis was performed using χ^2 test or Fisher exact test by comparing the general variations of copy numbers (ie, from 0 to > 3 copies). *P* values less than .05 were considered statistically significant.

RNA Extraction and Quantitative RT-PCR

Total RNA was isolated from tissues by using AxyPrepTM Blood Total RNA MiniPrep Kit (Axygen) according to the manufacturer's instruction. First strand cDNA was synthesized with RevertAidTM First Stand cDNA Synthesis Kit (Fermentas). The relative expression level of EphA3 mRNA was calculated by using the comparative C(T) method with RNAse P as the internal control.[18] Then the average expression level of EphA3 mRNA in the normal control samples with 2 copies of DNA was calculated. Fold change of each sample was presented as follows: fold change = relative expression level/average expression level in the normal control samples with 2 copies of DNA. Statistical analysis was performed using Student *t* test. *P* values less than .05 were considered statistically significant.

Results

Table 1 shows CNVs of EphA3 in hematologic malignancies and healthy controls. A total of 617 samples were examined. Statistical differences were observed in all six types of hematologic malignancies as compared with the controls ($P < .05$). Interestingly, the distribution of the copy numbers was not consistent among different types of malignancies. EphA3 was mostly deleted in the samples from AML, ALL, CML, CLL, MM, and MDS, and was amplified in rare cases (a maximum of 6 of 72 for CLL). However, CNVs of EphA3 in the M2 subtype (myeloblastic, with maturation) of AML didn't show significant difference with controls ($P = .100$). We then compared the CNVs among different types of malignancies, and found that much higher frequencies of CNVs existed in CML, CLL, MM, and MDS than that in AML ($P < .05$).

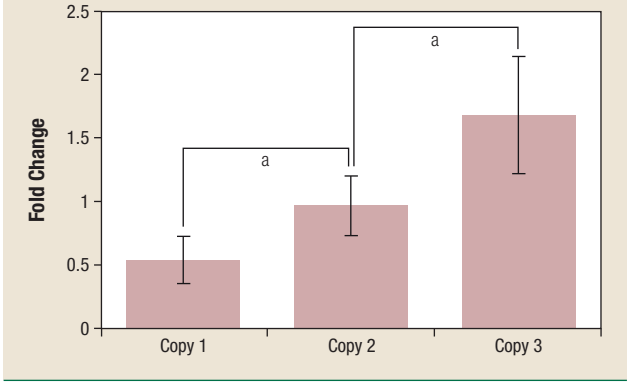
Gene CNVs can contribute to qualitative and quantitative diversities to their gene products. Next, we examined the tumor samples with 1, 2, or 3 copies of DNA, and tested whether the expression levels of EphA3 mRNA in these samples were correlated with the copy numbers. We selected the normal control samples with 2 EphA3 copies (n = 99), and the mean expression level from these samples was used for normalization (the average fold change in these samples was set as 1). The fold change of expression in tumor samples was calculated as follows: fold change = relative expression level/average expression level in the normal control samples with 2 copies of DNA (as shown in "materials and methods"). Reproducibility of this assay was established by running 5-fold replicate assays on small number of specimens. Representative results

Table 1 Copy Number Variants of EphA3 in Controls and Hematologic Malignancies

CNVs, Population	Number of Patients	Copy Numbers					*P* Value (vs. Control)	*P* Value (vs. AML)
		Deletion		2	Amplification			
		0	1		3	> 3		
Control	102	0	2	99	1	0	–	–
AML	114	1	17	90	4	2	.002	–
M2	47	0	4	41	1	1	.100	–
M5	17	0	3	13	1	0	.004	–
APL	22	1	5	14	1	1	2.2E-05	–
Others	28	0	5	22	1	0	.002	–
ALL	86	1	21	60	3	1	1.9E-05	.552
CML	78	2	34	37	4	1	3.9E-12	1.6E-04
CLL	72	1	31	34	4	2	5.7E-12	3.2E-04
MM	67	1	22	41	3	0	4.5E-08	.049
MDS	98	3	41	51	2	1	3.2E-11	2.4E-04

Abbreviations: ALL = acute lymphoblastic leukemia; AML = acute myelogenous leukemia; APL = acute promyelocytic leukemia; CLL = chronic lymphocytic leukemia; CML = chronic myelogenous leukemia; CNVs = copy number variants; MDS = myelodysplastic syndrome; MM = multiple myeloma

Figure 1 Expression of EphA3 mRNA in Tumor Samples With 1, 2, or 3 Copies of DNA



Thirty tumor samples (10 samples for 1, 2, or 3 EphA3 copies respectively) with similar percentage of neoplastic infiltrate were selected. The relative expression level of EphA3 mRNA was calculated by using the comparative C(T) method with RNAse P as the internal control. Fold change of each sample was calculated as follows: fold change = relative expression level / average expression level in the normal control samples with 2 copies of DNA. Y axis indicates the fold change of mRNA levels in each group. *P* value was calculated by Student *t* test.
[a]*P* < .05.

of the fold change were shown in Figure 1. A positive correlation of the EphA3 copy numbers with the expression of EphA3 mRNA was observed.

Discussion

Copy number variations have been clearly shown to have the potential to directly or indirectly influence a healthy individual's susceptibility to cancer, for example by varying the gene dosage of tumor suppressors or oncogenes.[35,36] However, there are many discrepancies among previous studies which used high-resolution approaches to screen CNVs.[29-33] Thus, validation of such CNVs by a large number of clinical samples is required. It is suggested that the genes present in very small regions of CNVs are excellent candidates for evaluation in cancer pathogenesis. Examination of the CNVs for such genes helps to understand the functional consequences of these CNVs. A previous study has shown EphA3 was deleted in AML samples.[11] But, in our study, we found that CNVs of EphA3 were also present, even with much higher frequencies, in other hematologic malignancies including ALL, CML, CLL, MM, and MDS. This tells us that the association with CNVs of EphA3 might be a common feature of hematologic malignancies. However, we didn't find significant difference for the M2 subtype (myeloblastic, with maturation) of AML (*P* = .100). This may be due to some unknown characters of the M2 subtype. But, more likely, it is because of the insufficient sample size (n = 47) of M2 subtype. So, in the future, larger sample size (eg, n = 100-200) is needed to further verify the association between M2 AML and CNVs of EphA3. It is worth mentioning that the frequencies of CNVs were relatively lower in acute leukemia (*P* = .002 and 1.9E-05 in AML and ALL, respectively) than in other malignancies (*P* values ranging from 4.5E-08 to 3.9E-12), suggesting that other factors, such as epigenetic alterations, may contribute to the development and progression of acute leukemia. However, all these diseases have varying pathogenesis and clinical features, making comparisons between these groups of disease quite difficult. In the future, it will be more helpful to focus on one disease entity which can allow for more directed clinical and biologic correlations/associations to be made. In our current study, we did not perform cell sorting for clonal cells of interest because of technique limitation during sample collection and handling. Cell sorting is important because the quantification of CNVs may be influenced by the issue of clonality within samples if the CNVs are somatic events. Sorting of clonal cells may help determine the true copy number of the sample. So, in the future, it would be conceivably of benefit to perform cell sorting to further validate our findings. Also, our current study is more descriptive and does not take into account clinical or translational relevance

in the diseases of concern. Correlating the findings of CNVs to parameters like survival, treatment responses, and other known prognostic factors (eg, correlating EphA3 CNV in patients with AML to Flt-3 ITD/TKD mutations, WBC count, age, NPM-1 mutations etc.) will be much helpful. We plan to collect samples with more detailed clinical information, hoping to explore the correlation of EphA3 CNVs with clinical parameters in the future.

It is expected that the CNVs do indeed have phenotypic consequences. Phenotypic effects of genetic differences, such as CNVs, are supposedly brought about by changes in expression levels.[37,38] We investigated the correlation between the expression of EphA3 mRNA and the copy numbers of its DNA. A positive correlation was observed. And this is consistent with two recent reports that assessed an overrepresentation of differentially expressed genes among CNV-mapping transcripts, and observed a weak yet significant positive correlation between relative expression level and gene dosage.[39,40] Moreover, the relatively high frequency of deletions of a single copy of the EphA3 locus in these malignancies suggests that there may be selection for either lower gene dosage, or for loss of heterozygosity. The expression data suggests that the remaining allele is certainly not silenced (as the remaining alleles may be in some tumor suppressor genes). In the future, it would be of great interest to know whether the remaining allele has a germline or mutated sequence.

In general, plausibly, the CNVs of EphA3 have the potential to serve as a diagnostic indicator, alone or in combination with other markers, for hematologic malignancies. However, the functional consequences of CNVs and the underlying mechanisms of the heterogeneous expression levels need to be extensively investigated in the future.

Conclusion

The CNVs of EphA3 were found to associate with multitypes of hematologic malignancies including ALL, AML, CLL, CML, MM, and MDS. The mRNA levels of EphA3 were weakly yet positively correlated with the copy numbers of EphA3. Thus, CNVs of EphA3 might be a diagnostic indicator for hematologic malignancies. In the future, the functional consequences of EphA3 CNVs need to be more extensively studied.

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Disclosures

The authors have no relevant relationships to report.

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