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Advances in Brief

Specific *c-kit* Mutations in Sinonasal Natural Killer/T-Cell Lymphoma in China and Japan[1]

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Abstract

Sinonasal lymphoma is one of the constituents of lethal midline granuloma, which is a clinical term for progressive, destructive lesions affecting the midline of the face. The majority of sinonasal lymphomas, especially those showing polymorphous patterns of proliferation and thus termed polymorphic reticulosis, recently were categorized as sinonasal natural killer/T-cell lymphomas. They are more prevalent in Asia than Europe or North America and are associated with EBV infection. Twenty-three cases with sinonasal natural killer/T-cell lymphomas were collected from two high-incidence regions: Beijing, China (14 cases) and Osaka, Japan (9 cases). *c-kit* mutations were analyzed on paraffin-embedded specimens by PCR-single-strand conformation polymorphism followed by direct sequencing; the *c-kit* proto-oncogene encodes a receptor of tyrosine kinase, which plays an important role in the regulation of normal and neoplastic hematopoiesis by the interaction with its specific ligand, termed stem cell factor. Twelve single nucleotide substitution mutations were seen in 23 cases. Ten of 14 Chinese cases (71.4%) had mutations at exon 11 or exon 17, whereas only two of nine Japanese cases (22.2%) had mutations, showing a significant difference in frequency between Chinese and Japanese cases. Furthermore, seven of eight mutations (92%) in exon 17 occurred at codon 825 and three of four mutations (75%) in exon 11 occurred at codon 561. Such a specificity has not been reported before, and these results, taken together, suggest that location-specific differences in etiological factors cause specific mutations in *c-kit* gene.

Introduction

Sinonasal NKTCL[3] is a clinical condition of lethal midline granuloma that shows necrotic and granulomatous lesions in the upper respiratory tract, especially in the nasal cavity. The disease is more frequent in Asian countries such as China, Korea, and Japan than in Western countries (1, 2). Histologically, the disease shows a polymorphous pattern of proliferation consisting of large atypical cells, small lymphocytes, macrophages, and plasma cells. Thus, the term polymorphic reticulosis was given to this disease (3). Polymorphic reticulosis was recently categorized as sinonasal NKTCL (4) and was demonstrated to be highly associated with EBV infection (5, 6). However, molecular study of the disease has not been carried out because massive necrosis makes it is difficult to get living tumor tissues.

The *c-kit* proto-oncogene encodes a receptor of tyrosine kinase, which is essential to hematopoiesis, and recently, the development of acute leukemia or malignant lymphoma was reported in transgenic mice expressing KIT^{V814} (Asp814→Val; Ref. 7). Consequently, sinonasal NKTCLs in two high incidence regions of Beijing (China) and Osaka (Japan) were examined for the *c-kit* gene mutation.

Materials and Methods

Cases. Twenty-three cases (21 males and 2 females) with sinonasal lymphoma, 14 from China and 9 from Japan, were selected for this study. Patient ages ranged from 18 to 78 years (median, 46.9 years). Patients were admitted to hospitals during the period of 1986–1997 (Table 1), and biopsy of the tumor was done for histological diagnosis. All patients had necrotic and granulomatous lesions in the upper respiratory tract. The nasal cavity and paranasal sinuses were the predominant sites (19 cases) for involvement, followed by the naso-/oro-pharynx (2 cases), tonsils (1 case), and trachea (1 case). For histological studies, specimens were fixed in 10% buffered or neutral formalin and embedded in paraffin. Tumor tissues sections (3 μm) were prepared and stained with H&E and immunoperoxidase procedures (avidin-biotin-peroxidase complex method) using CD3 (Dakopatts, Glostrup, Denmark), MT-1 (CD43; BioScience, Gmmenbrucke, Switzerland), MX-PanB (CD20; Kyowa Medex, Tokyo, Japan), 123C3 (CD56; Zymed, South San Francisco, CA), ZH7 (CD16; Novocastra, Newcastle, United Kingdom), and TIA-1 (Coulter, Hialeah, FL).

Detection of *c-kit* Mutations. DNA was extracted using chelating resin for PCR amplification. In brief, three 10-μm thick paraffin sections were cut, transferred into sterile distilled water containing 20% chelating resin (iminodiacetic acid; Sigma, St. Louis, MO), and boiled for 15 min. After centrifugation, the supernatant was transferred to a sterile 500-μl tube and stored at −20°C.

The PCR primer pairs for the amplification of the *c-kit* gene exons 11 and 17 were as follows: 5′-GATCTATTTTTCCCTTTCTC-3′ and 5′-AGCCCCTGTTTCATACTGAC-3′ for exon 11; 5′-CATGGTCGGATCACAAAGAT-3′ and 5′-ATTATGAAAGTCACGGAAAC-3′ for exon 17. DNA amplification and nonradioactive SSCP (Cold SSCP) analysis were carried out to detect mutations as described previously (8, 9). The mutated SSCP bands were extracted from the gel and reamplified by PCR for 25 cycles to enrich mutated alleles.

Sequencing was performed by the dideoxy chain termination method using the Big Dye terminator cycle sequencing kit (Perkin-Elmer, Foster City, CA). Sequencing primers were the same as those used for PCR. Cycle sequencing was performed following the protocol, *i.e.*, 30 cycles of denaturation (95°C for 30 s), annealing (54°C for 30 s) and extension (72°C for 3 min) followed by cooling at 20°C after the final cycle. After ethanol precipitation, the samples were analyzed by the Genetic Analyzer (ABI PRISM 310; Perkin-Elmer, Foster City, CA). PCR-SSCP analyses and sequencing of mutated bands were repeated three times for each sample to rule out the possibility of contamination and PCR fidelity artifacts. As controls, *c-kit* mutations in peripheral blood mononuclear cells were examined in 10 healthy Japanese and 10 Chinese (12 males and 8 females; median age, 30 years; range, 24–47 years) living in Osaka.

Site-directed Mutagenesis and Transfection. To directly examine the causal role of the *c-kit* mutations in activation of *c-kit* tyrosine kinase activity, site-directed mutagenesis was performed using murine *c-kit* cDNA as described previously (10). Briefly, the expression vectors containing wild-type and mutated *c-kit* cDNA [Ala-823, which was constructed by site-directed mutagenesis to correspond to human *c-kit*R (Ala-825)] were transfected into

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[3] The abbreviations used are: NKTCL, natural killer/T-cell lymphoma; SSCP, single-strand conformation polymorphism; mAb, monoclonal antibody.

Table 1 *c-kit mutations in sinonasal NKTCL patients in China and Japan*

			c-kit mutation					
			Exon 11			Exon 17		
Patient no.	Age (years)	Sex	Codon	Nucleotide	Amino acid	Codon	Nucleotide	Amino acid
Beijing, China								
1	53	M	561	GAG → GAA	Glu → Glu		None	
2	47	M		None[a]		816	GAC → AAC	Asp → Asn
3	22	M		None		825	GTT → GCT	Val → Ala
4	34	M		None			None	
5	57	M		None		825	GTT → GCT	Val → Ala
6	67	M		None			None	
7	18	M		None		825	GTT → GCT	Val → Ala
8	31	M		None			None	
9	28	M		None		825	GTT → GCT	Val → Ala
10	39	M		None		825	GTT → GCT	Val → Ala
11	40	M		None			None	
12	42	M	559	GTT → ATT	Val → Ile		None	
13	32	M	561	GAG → AAG	Glu → Lys		None	
14	33	M		None		825	GTT → GCT	Val → Ala
Osaka, Japan								
1	78	F	561	GAG → AAG	Glu → Lys		None	
2	71	M		None			None	
3	60	M		None			None	
4	71	M		None			None	
5	48	M		None			None	
6	57	M		None			None	
7	52	M		None			None	
8	50	F		None			None	
9	49	M		None		825	GTT → GCT	Val → Ala
Controls[b]								
1–20	24–47	M&F		None			None	

[a] Mutation not detected.
[b] Peripheral blood mononuclear cells from healthy Chinese (6 males and 4 females) and Japanese (5 males and 5 females).

the human embryonic kidney cell line 293T by the calcium phosphate method. The cells were cultured in DMEM containing 10% FCS and fed every 3 days with fresh medium. To determine the expression of *c-kit* product in the transfected 293T cells, the transfectants were incubated with [^{35}S]methionine (3.7×10^6 Bq/ml; DuPont/NEN research Products) for 6 h and lysed with NP 40 lysis buffer. The labeled *c-kit* product was then immunoprecipitated with a rat antimouse *c-kit* mAb (mAb ACK-2; kindly provided by Dr. S-I. Nishikawa, Kyoto University, Kyoto, Japan) and analyzed by SDS-PAGE and autoradiography. To examine the activation and tyrosine phosphorylation of *c-kit* product, the *c-kit* product of each transfectant was immunoprecipitated with rabbit antiserum against murine *c-kit* protein from the lysates, and then subjected to *in vitro* immune complex kinase assay and immunoblotting with an antiphosphotyrosine mAb as described above.

Results and Discussion

Histologically, varying degrees of necrosis was found in the upper respiratory lesions. Diffuse proliferation of large atypical mono- or multinucleated cells was observed with various numbers of lymphocytes, plasma cells, and macrophages, giving a polymorphous appearance. The immunohistochemical findings of 23 cases were in agreement with those of sinonasal NKTCL, *i.e.,* TIA-1$^+$, CD56$^+$, CD3$^+$, and/or CD43$^+$.

As shown in Table 1, 10 of 14 Chinese sinonasal lymphoma cases (71.4%) showed mutations at exon 11 (3 cases, 21.4%) and exon 17 (7 cases, 50.0%), whereas only 2 of 9 Japanese cases (22.2%) had mutations at exon 11 (1 case, 11.1%) and exon 17 (1 case, 11.1%; $P < 0.05$ by Fisher's exact test). Furthermore, mutations occurred at highly restricted sites. Seven of eight *c-kit* mutations in exon 17 occurred at codon 825 (GTT→GCT; Val→Ala), and three of four mutations in exon 11 occurred at codon 561 [two GAG→AAG (Glu→Lys); one silent GAG→GAA (Glu→Glu)]. All of 12 mutations were caused by single-nucleotide substitutions. Among them, 11 were missense mutations leading to amino acid substitution, and the other was a silent mutation without any amino acid changes. Seven of the 12 (58.3%) single-nucleotide substitution were A:T to G:C transitions, and the remainders (41.7%) were G:C to A:T transitions. Transversion was not detected in the present cases, and no mutations involved CpG dinucleotides.

The *c-kit* gene is the cellular homologue of the viral oncogene, and several studies have suggested that a mechanism of stromal/parenchymal interactions via the *c-kit* receptor-ligand system might be involved in normal hematopoiesis, gametogenesis, and melanogenesis (11–13). In the present study, 10 of 12 *c-kit* mutations were detected at codons 825 and 561. Such hot spots were not reported in any of the earlier studies of human and animal neoplasms. However, mutations of the *c-kit* gene at codon 816 are known to cause substitution of tyrosine or valine for aspartate in mast cells derived from patients with mastocytosis and urticaria pigmentosa (14, 15). The protein product of the *c-kit* gene is a tyrosine kinase receptor that consists of four parts, *i.e.*, the extracellular, transmembrane, juxtamembrane, and tyrosine kinase domains. Both codons 816 and 825 are located on the tyrosine kinase domain. A mutation in codon 560 (Val→Gly) on the juxtamembrane domain was also reported to cause constitutive phosphorylation and activation of KIT in human gastrointestinal stromal tumors (10, 16–18). These mutations can transform cell lines from factor-dependent growth to factor-independent growth (16, 18–20).

To determine whether KIT^{A825} is a gain-of-function mutation, a mutated murine *c-kit* gene encoding murine *c-kit*R (Ala-823) was constructed by site-directed mutagenesis corresponding to human *c-kit*R (Ala-825) and transfected into a human embryonic kidney cell line. In the transfected cells, wild-type *c-kit*R and mutant *c-kit*R (Ala-823) were neither phosphorylated on tyrosine nor activated in an immunocomplex kinase reaction in the absence of stem cell factor. KIT^{A825}, frequently observed in our cases with sinonasal NKTCL, is not a gain-of-function mutation.

In the present study, analysis of sinonasal NKTCL from China and Japan shows that the occurrence of specific *c-kit* substitution muta-

tions and the spectrum of *c-kit* mutations (codons 651 and 825) are very similar in China and Japan. However, the mutation frequency was significantly different between the two Asian populations. The difference may be caused by the migration of susceptible populations or some environmental confounding factors that produce specific mutations. Additional studies on the *c-kit* and other mutations in China and Japan will be necessary to understand the difference in oncogenesis of NKTCL.

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