Gene mutation analysis of sinonasal lymphomas in Indonesia

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Abstract. Sinonasal lymphomas comprise NK/T-cell (NKTCL) type and B-cell type with unique geographical development. In this study, mutations of *p53*, K-*ras*, c-*kit*, *ß-catenin*, and *bak* gene were analyzed using polymerase chain reaction (PCR)-single strand conformation polymorphism (SSCP) followed by direct sequencing in 41 sinonasal lymphomas (27 NKTCL and 14 B-cell type) from Indonesia. *In situ* hybridization study with EBER-1 probe revealed that 85% of NKTCL cases were EBV positive, but none of B-cell type was EBV positive. Frequency of mutations in *p53*, K-*ras*, c-*kit*, *ß-catenin*, and *bak* gene was 62.9%, 0%, 11.1%, 18.5%, and 25.9%, respectively, in NKTCL, and 71.4%, 0%, 23.1%, 21.4%, and 57.1%, respectively, in B-cell cases, showing that mutation frequency in all genes was higher in B-cell than in NKTCL cases. These findings suggest that gene mutations might be the driving-force for B-cell lymphoma, whereas combined EBV infection and gene mutations contribute to NKTCL development in Indonesia.

Introduction

Sinonasal lymphoma usually presents as lethal midline granuloma, which is a clinical condition of progressive and destructive lesions affecting the midline of the face, especially nasal cavity (1,2). Immunophenotypically, sinonasal lymphoma could be divided into NK/T-cell (NKTCL) type which shows a polymorphous pattern of proliferation consisting of numerous inflammatory cells and B-cell type which shows a monomorphous proliferation of tumor cells. Clustering of patients with sinonasal NKTCL is found in Asian (3,4) and Central and South American countries (5), while B-cell lymphoma is the commonest type of lymphoma in the sinonasal region in Western countries (6,7). These findings might suggest a causative role of some genetic, environmental, and lifestyle factors for human nasal lymphomagenesis.

We have reported higher frequency of *p53* mutations in sinonasal NKTCL cases in Japan (8) and North China (9) compared to in Korea (10). p53 is a tumor suppressor gene which causes cells with damaged DNA to arrest at the G1 phase or stimulates the expression of BAX, the pro-apoptotic protein. A previous study revealed that p53 gene mutations occur mostly in exon 4 to 8 in various kinds of human malignancies (11). High incidence of malignant lymphoma in the *p53* knock out mice has been reported (12), suggesting an important role of *p53* gene mutation in lymphomagenesis.

The c-*kit* proto-oncogene encodes a tyrosine kinase receptor which plays an important role in proliferation and differentiation of hematopoietic stem cells, mast cells, and interstitial cells of Cajal (13). Previous study showed a relatively high frequency of c-kit mutations among nasal NKTCL cases in China but not in Japan and Korea (14).

The K-*ras* gene encodes a protein, referred to as p21, which plays a role in signal transduction through transmembrane signaling system (15). K-*ras* mutations are frequently found in pancreatic, colorectal and lung malignancies, but is rare in lymphomas (16). Our recent study also showed that mutations of K-*ras* were uncommon in nasal NKTCL cases in east Asian countries (9).

ß-catenin functions downstream of the Wnt signaling pathway, and activated ß-catenin complex leads to overexpression of c-*myc*, which regulates cell proliferation (17,18). Mutations of *ß-catenin* were found in a relatively high frequency in nasal NKTCL cases in Japan (9).

bak, a member of the Bcl-2 family, functions as a tumor suppressor gene through binding and inhibiting the anti-apoptotic molecule Bcl-xL, thereby inducing apoptosis (19). Mutations of the *bak* gene are relative frequently found in advanced stage of cervical, gastric, and colorectal cancers (20,21).

In the present study, mutations of *p53*, K-*ras*, c-*kit*, *ß-catenin*, and *bak* gene were examined on paraffin-embedded tissues from 41 Indonesian patients with sinonasal

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Key words: sinonasal lymphoma, single strand conformation polymorphism, mutation, epidemiology

Table I. Oligonucleotide primers used for PCR.

Gene	Exon		Sequences	Ta (˚C)	Tm (˚C)
p53	4a	F	5'-TTTTCACCCATCTACAGTCC-3'	58	20
		R	5'-CAAGAAGCCCAGACGGAAAC-3'		
	4b	F	5'-CCTGGCCCCTGTCATCTTCT-3'	58	20
		R	5'-AAGAAATGCAGGGGGATACG-3'		
	5a	F	5'-TCTGTCTCCTTCCTCTTCCTA-3'	57	35
		R	5'-CATGTGCTGTGACTGCTTGT-3'		
	5b	F	5'-TGTGCAGCTGTGGGTTGATTC-3'	62	25
		R	5'-CAGCCCTGTCGTCTCTCCAG-3'		
	6	F	5'-TTGCTCTTAGGTCTGGCCCCT-3'	64	35
		R	5'-TAGGGAGGTCAAATAAGCAG-3'		
	7	F	5'-TAGGTTGGCTCTGACTGTACC-3'	60	25
		R	5'-TGACCTGGAGTCTTCAGTGT-3'		
	8	F	5'-TCTTGCTTCTCTTTTCCTAT-3'	56	10
		R	5'-CGCTTCTTGTCCTGCTTGCT-3'		
K-*ras*	1	F	5'-CATGTTCTAATATAGTCACA-3	48	25
		R	5'-CTCTATTGTTGGATCATATTCGTCC-3'		
	2	F	5'-ACTGTGTTTCTCCCTTCTCA-3'	48	5
		R	5'-CACAAAGAAAGCCCTCCCCA-3'		
c-*kit*	11	F	5'-GATCTATTTTTCCCTTTCTC-3'	56	20
		R	5'-AGCCCCTGTTTCATACTGAC-3'		
	17	F	5'-CATGGTCGGATCACAAAGAT-3'	54	15
		R	5'-ATTATGAAAGTCACGGAAAC-3'		
ß-catenin	3	F	5'-GCTGATTTGATGGAGTTGGA-3'	56	25
		R	5'-GCTACTTGTTCTTGAGTGAA-3'		
bak	3	F	5'-TGCCTCCCTGAAGATGTCCT-3'	60	25
		R	5'-TGACTCCCAGCTTTGATCCT-3'		
	4	F	5'-GGCAGGGTATGGTATGGTTG-3'	60	20
		R	5'-TCCCGACTGCCTGGTTACTG-3'		
	6	F	5'-GCAAGGGAACAGAGAAGGCA-3'	60	25
		R	5'-TGACCACCTTGTTTCTCCCG-3'		

Ta, annealing temperature for DNA amplification; Tm, maintaining temperature of circulating buffer during electrophoresis for 'cold SSCP' analysis.

lymphomas by PCR-single strand conformation polymorphism (SSCP) followed by direct sequencing.

Materials and methods

Case selection. Forty-nine cases with sinonasal lymphoproliferative diseases which were registered in the Department of Anatomical Pathology, Faculty of Medicine, University of Indonesia/Dr. Cipto Mangunkusumo Hospital, Jakarta during the period 1994 to 2004 were retrieved for the current study. Biopsy specimens obtained from the sinonasal lesions of these cases were fixed in 10% formalin and routinely processed for paraffin-embedding. Histologic sections, cut at 4 μm, were stained with hematoxylin and eosin and immunoperoxidase procedures (avidin-biotin-peroxidase method). Diagnosis of sinonasal lymphomas, whether NKTCL or B-cell type, was made based on histologic and immunohistochemical findings. Forty-three cases were diagnosed as sinonasal lymphoma, while the biopsy specimen in the remaining 6 cases did not contain representative lesions. These 6 cases together with two from which extraction of adequate amount of DNA was not possible were excluded from this study. Information for age and gender were not available in 5 cases. Histologic and immunohistochemical findings together with the brief clinical findings of a part of these cases were reported previously (22). Briefly, there was a marked male preponderance in NKTCL type as compared to B-cell type: 2.8 to 1 and 1 to 1.6, respectively. The mean age in NKTCL cases (37 years) was younger than that in B-cell cases (49 years). Immunohistochemical study revealed that 27 (65.9%) cases were NKTCL ($CD3^+$, $CD4^+$, $CD8^+$, $CD45RO^+$, $CD56^+$, and/or TIA-1^+) and 14 (34.1%) diffuse large B-cell lymphoma ($CD20^+$, $CD3^-$, $CD56^-$). *In situ* hybridization study using EBER-1 probe revealed EBV genome in the nucleus of tumor cells in 23 of 27 (85%) cases of NKTCL, but none of B-cell lymphoma.

Table II. Results of gene mutation analysis.

Case		Age	Sex	*p53*			c-*kit*		
				Exon/codon	Nucleotide	Amino acid	Exon/codon	Nucleotide	Amino acid
NKT-cell	1	38	M	4/58	CCA→CAA	Pro→Gln			
				4/118	ACA→ATA	Thr→Ile			
	2	38	M						
	3	48	M						
	4	22	M	4/116	TCT→TTT	Ser→Phe			
				Intron 4	12317 G→A				
				5/151	CCC→TCC	Pro→Ser			
	5	46	M	4/111	CTG→CCG	Leu→Pro			
				Intron 4	12307 G→A				
				Intron 4	12314 G→A				
	6	28	F	4/99	TCC→TTC	Ser→Phe			
	7	50	M	5/184	GAT→AAT	Asp→Asn			
	8	48	M						
	9	36	M						
	10	54	M						
	11	17	M	7/250	CCC→CTC	Pro→Leu			
	12	36	M	8/283	CGC→CAC	Arg→His			
	13	62	F						
	14	51	F	4/47	CCG→CTG	Pro→Leu			
	15	39	M	5/148	GAT→AAT	Asp→Asn			
	16	16	M						
	17	67	M						
	18	12	M	4/47	CCG→TCG	Pro→Ser			
	19	24	M	4/100	CAG→TAG	Gln→stop			
				5/155	ACC→ATC	Thr→Ile			
	20	6	F				11/577	CCT→TCT	Pro→Ser
	21	N/A		5/153	CCC→TCC	Pro→Ser	17/825	GTT→GCT	Val→Ala
				5/160	ATG→ACG	Met→Thr			
				6/219	CCC→CGC	Pro→Arg			
				7/252	CTC→TTC	Leu→Phe			
	22	N/A		4/118	ACA→GCA	Thr→Ala			
				5/187	GGT→GAT	Gly→Asp			
				7/248	CGG→GGG	Arg→Gly			
				8/299	CTG→GTG	Leu→Val			
	23	48	F	5/153	CCC→TCC	Pro→Ser			
				7/254	ATC→GTC	Ile→Val			
				8/298	GAG→AAG	Glu→Lys			
	24	41	M	4/76-90 deletion			11/576	CTT→CCT	Leu→Pro
				Intron 7	Nucleotide 14117 A→G		Intron 17	Nucleotide 7217 A→C	
	25	35	F						
	26	N/A		5/173	GTG→ATG	Val→Met			
	27	N/A		4/72	CGC→TGC	Arg→Cys			
B-cell	1	60	F	5/166	TCA→CCA	Ser→Pro			
	2	57	M	4/121	TCT→TTT	Ser→Phe			
				5/144	CAG→CGG	Gln→Arg			
				5/147	GTT→ATT	Val→Ile			
	3	67	F	4/52	CAA→TAA	Glu→stop			
				4/66	ATG→ATA	Met→Ile			
				4/76-90 deletion					
	4	76	F	5/146	TGG→TAG	Trp→stop			
	5	70	M	4/76-90 deletion			11/566	AAC→AAT	Silent
				4/109	TTC→TTT	Silent			
				8/267	CGG→CGC	Silent			

Table II. Continued.

Case	Age	Sex	*p53*			c-*kit*		
			Exon/codon	Nucleotide	Amino acid	Exon/codon	Nucleotide	Amino acid
6	63	M	4/108	GGT→AGT	Gly→Ser	11/561	GAG→AAG	Glu→Lys
			5/150	ACA→ATA	Thr→Ile			
			Intron 7	14151 C→T				
7	30	F	4/94	TCA→ACA	Ser→Thr			
			Intron 4	12302 C→T				
			5/168	CAC→TAC	His→Tyr			
8	16	F	4/100	CAG→TAG	Gln→stop	11/564	AAT→AGT	Asn→Ser
			Intron 4	12317 G→A				
			8/266	GGA→GAA	Gly→Glu			
9	40	F						
10	50	F						
11	6	M	5/187	GGT→AGT	Gly→Ser			
12	49	M						
13	52	F	5/133	ATG→ACG	Met→Thr			
			5/145	CTG→CCG	Leu→Pro			
14	N/A							

Case	*ß-catenin*			*bak*		
	Exon/codon	Nucleotide	Amino acid	Exon/codon	Nucleotide	Amino acid
NKT-cell	1			3/28	GCC→GTC	Ala→Val
				3/60	ATG→ACG	Met→Thr
				6/187	CCC→TCC	Pro→Ser
				6/n851	C→T	
	2 3/48	GGT→GAT	Gly→Asp			
	3					
	4					
	5			6/187	CCC→TCC	Pro→Ser
				6/n851	C→T	
	6 3/31	CTG→TTG	Silent			
	3/53	GAG→AAG	Glu→Lys			
	7					
	8			3/30	GAC→AAC	Asp→Asn
				3/45	CAG→CAA	Silent
				6/n 839	C→T	
	9 3/43	GCT→GTT	Ala→Val			
	3/51	AAT→AGT	Asn→Ser			
	10 3/29	TCT→TCC	Silent			
	11 3/38	GGT→AGT	Gly→Ser	6/n 830	C→T	
	3/48	GGT→GAT	Gly→Asp			
	12					
	13					
	14					
	15					
	16					
	17					
	18			6/193	GTG→ATG	Val→Met
				6/196	GGT→GAT	Gly→Asp
	19			6/187	CCC→TCC	Pro→Ser
				6/197	GTG→ATG	Val→Met
				6/n 844	G→A	

Table II. Continued.

Case	*ß-catenin*			*bak*		
	Exon/codon	Nucleotide	Amino acid	Exon/codon	Nucleotide	Amino acid
	20					
	21					
	22					
	23					
	24			3/39	GTT→GGT	Val→Gly
	25					
	26					
	27 3/43	GCT→GTT	Ala→Val			
B-cell	1			6/n 844	G→A	
	2 3/46	CTG→CTA	silent	6/191	GTG→GCG	Val→Ala
				6/n 828	C→T	
				6/n 837	G→C	
	3					
	4 3/43	GCT→GTT	Ala→Val			
	3/48	GGT→GAT	Gly→Asp			
	5			3/39	GTT→GGT	Val→Gly
	6			6/205	1 bp deletion (frame shift)	
	7					
	8 3/44	CCT→CTT	Pro→Leu	6/n 861	C→T	
	9					
	10					
	11 3/23	AGT→GGT	Ser→Gly	3/59	GAG→GAA	Silent
	3/66	TGG→TAG	Trp→stop	6/190	AAC→AAT	Silent
				Intron 3	g4391 G→A	
				Intron 3	g4414 C→T	
	12					
	13			3/45	CAG→CGG	Gln→Arg
				3/67	CCT→CTT	Pro→Leu
				6/180	GCC→GCT	Silent
	14					
				6/186	GGT→GAT	Gly→Asp

NKT, natural-killer/T-cell; N/A, not available; F, female; M, male; g, genomic nucleotide number.

Detection of gene mutations. DNA was extracted from the paraffin-embedded specimens using chelating resin for PCR amplification. In brief, three 10 μm-thick paraffin sections were transferred into sterile distilled water containing 20% chelating resin and 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 exons 4 through 8 of p53, exons 1 and 2 of K-*ras*, exons 11 and 17 of c-*kit*, exon 3 of *ß-catenin* and, exon 3, 4 and 5 of *bak* are listed in Table I. DNA amplification and non-radioactive SSCP (cold SSCP) analysis were carried out to detect mutations as described previously (23). 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 (Applied Biosystems, Foster City, CA). Sequencing primers were the same as those used for PCR. Cycle sequencing was performed as follows: 30 cycles of denaturation (95˚C for 30 sec), annealing (54˚C for 30 sec), and extension (72˚C for 3 min) followed by cooling at 20˚C after the final cycle. After ethanol precipitation, the samples were analyzed with ABI PRISM 310 Genetic Analyzer. PCR-SSCP analysis and sequencing of mutated bands were repeated three times for each sample to rule out the possibility of contamination and PCR fidelity artifacts.

Results

Direct sequencing of SSCP products revealed the mutations of the *p53* gene in 17 of 27 cases (62.9%) of NKTCL and 10 of 14 cases (71.4%) of B-cell type (Tables II and III). Among 17 NKTCL and 10 B-cell cases with *p53* mutations, the mutations were more frequently found in exon 4 (10 of

Table III. Summary of mutations.

Immunophenotype	Number of cases	Gene mutation				
		p53	K-*ras*	c-*kit*	*ß-catenin*	*bak*
NK/T-cell	27	17 (62.9)	0 (0)	3 (11.1)	5 (18.5)	7 (25.9)
B-cell	14	10 (71.4)	0 (0)	3 (23.1)	3 (21.4)	8 (57.1)

NK/T-cell, natural-killer/T-cell; Numbers in parentheses represent percentage.

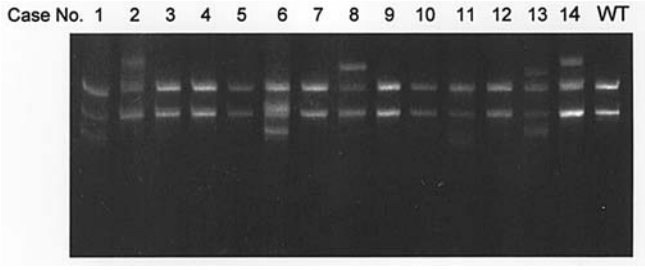

Figure 1. Cold SSCP analysis of sinonasal lymphomas in Indonesia. Half of 14 B-cell types of sinonasal lymphomas in Indonesia showed aberrant bands comparing with wild-type (WT) band in exon 6 of the *bak* gene.

17 NKTCL and 6 of 10 B-cell cases) and exon 5 (8 of 17 NKTCL and 7 of 10 B-cell cases), but much less frequently in exon 6 (1 of 17 NKTCL and none of 10 B-cell cases), exon 7 (4 of 17 NKTCL and none of 10 B-cell cases), and exon 8 (3 of 17 NKTCL and 2 of 10 B-cell cases). Twenty-six and 13 missense mutations were found in 16 NKTCL cases and 8 B-cell cases, respectively. Non-sense mutations were found in one and three of NKTCL and B-cell cases, respectively. Deletion of codon 76 to 90, which is located in exon 4 of p53, occurred in one and two of NKTCL and B-cell cases, respectively. Intronic mutations were found in three NKTCL and B-cell cases.

K-*ras* mutation was not found in any of the 41 sinonasal lymphomas examined. Missense mutations of c-*kit* gene were found in three NKTCL and two B-cell cases, whereas a silent mutation was found in one B-cell case. Intronic mutation was found in one NKTCL case. Seven and four missense mutations of *ß-catenin* occurred in 5 of 27 (18.5%) NKTCL cases and in 3 of 14 (21.4%) B-cell cases, respectively. Non-sense mutation of codon 66 of *ß-catenin* was found in one B-cell case, while silent mutations were found in two and one of NKTCL and B-cell cases, respectively.

Frequency of *bak* gene mutations in B-cell cases (57.1%) was higher than that in NKTCL cases (25.9%) (P=0.0527) (Fig. 1). Ten and five missense mutation of *bak* gene was found in 6 of 27 (22.2%) NKTCL cases and in 4 of 14 (28.6%) B-cell cases, respectively. Whereas silent mutations were found in one of NKTCL and two of B-cell cases. Single nucleotide substitution or deletion outside the open reading frame was found in five NKTCL and four B-cell cases, while intronic mutation near exon 3 was found in one B-cell case.

Discussion

Sinonasal lymphomas in Indonesia showed similar features to those in other Asian countries: predominance of NKTCL type with presentation of characteristic necrotic and granulomatous lesions in the upper respiratory tract, and a close association with EBV. The median age of patients with NKTCL (37 years) was rather close to that in North China (36.5 years) (8), Northeast China (40 years) (9), and Korea (41 years) (10), but much lower than that in Japan (61 years). Meanwhile none of sinonasal B-cell lymphoma of Indonesia was EBV-positive, and its median age at diagnosis (49 years) was rather high. These findings suggest the presence of different etiologic factors such as environmental agents and social habits for each type of sinonasal lymphomas by geographic areas.

The frequency of p53 mutations in the present cases with NKTCL (62.9%) was close to that in North China (63.2%) and Japan (62.1%), but higher than that in Korea (31%) and Northeast China (40%) (8,9). Northeast China locates near the boundary of Korean peninsula. The p53 mutation frequency in B-cell type (71.4%) was higher than that in NKTCL, but statistically not significant. Majority of mutations in NKTCL and B-cell sinonasal lymphoma in Indonesia was found in exons 4 and 5. The higher frequency of exon 4 involvement was also observed in Japanese (31.1%), but not in Korean (7.1%) and Northeast China (10%) cases of NKTCL (8,9). These findings showed that mode of *p53* mutations in Indonesian cases of sinonasal lymphoma was similar to that in Japan. In the current cases, majority of the mutations were single nucleotide substitutions, comprising missense mutations in 16 (59.3%) NKTCL and 8 (57.1%) B-cell cases, and non-sense mutations in 1 (3.7%) NKTCL and 3 (21.4%) B-cell cases.

Mutations of *ras* (H, K, and N-*ras*) were uncommon in lymphomas developing in immunocompetent patients, whereas relatively high frequency of K-*ras* mutations have been reported in lymphomas of immunocompromised patients (16,24). In Indonesian sinonasal lymphoma cases, K-*ras* mutations were not observed. K-*ras* mutations are exceptional among sinonasal NKTCL in Asian countries.

Frequency of c-*kit* mutations was slightly higher in B-cell (23.1%) than in NKTCL of Indonesian cases (11.1%). The frequency in NKTCL was close to that in Korea (11.9%), Japan (5.5%), Northeast China (5%), but was far different from that in North China, where the frequency of c-*kit*

mutation was extraordinarily high (71.4%) (9,14). Some factors present in North China might induce c-*kit* mutations. In this regard, analysis of cases from other parts of China than North and Northeast will give some clues. The mutation frequency of ß-catenin in B-cell cases (21.4%) was rather similar to that in NKTCL cases (18.5%).

This is the first time that *bak* gene was examined in gene mutation analysis of NKTCL in an Asian country. *bak* is an apoptosis-inducer tumor suppressor gene; its mutations were frequently found in the cervical (14.3%), gastric (12.5%), and colorectal (10%) cancers of advanced stage but rarely studied in lymphomas (20,21). The frequency of *bak* mutations was higher in B-cell (57.1%) than in NKTCL (25.9%) type (P=0.0527). This finding suggests that anti-apoptosis event induced by *bak* mutation might play a role for the development of sinonasal lymphoma of B-cell type.

In conclusion, the frequency of mutations was higher in *p53*, c-*kit*, and *bak* genes examined in the B-cell than in NKTCL cases, with almost statistically significant level in *bak* gene. This contrast the higher frequency of EBV positive rate in NKTCL cases but none of B-cell cases were EBV positive among Indonesian sinonasal lymphomas (22). Taken together, gene mutations might be the driving-force for development of B-cell lymphoma, whereas combined EBV infection and gene mutations might contribute to NKTCL development in Indonesian sinonasal lymphomas. EBV positive rate in sinonasal B-cell lymphoma in Japan was 40% (25). These findings suggest a causative role of genetic, environmental, and life style factors for human nasal lymphomagenesis.

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