N-*ras* mutations in myelodysplastic syndromes in Hong Kong Chinese

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Hong Kong Chinese patients with myelodysplastic syndromes were screened for the presence of N-*ras* mutation by using the polymerase chain reaction/dot blot hybridization technique. Three of 20 (15%) cases studied were found to harbour mutant N-*ras* in the bone marrow mononuclear cell population. One case of refractory anaemia with excess of blasts and with excess of blasts in transformation had substitution of arginine for glutamine at codon 61. A second case of refractory anaemia with excess of blasts had substitution of serine for glycine at codon 12. In this patient, mutant N-*ras* was detected in a follow up sample taken two months later, although there was no change in the blast percentage compared with the presentation sample. Our study showed that N-*ras* mutation is an infrequent finding in Hong Kong Chinese myelodysplastic syndrome patients. An N-*ras* mutation is not necessarily accompained by an alteration in the haematological picture.

HKMJ 1995;1:290-294

Key words: Mutation; Genes, ras; Myelodysplastic syndromes; Hong Kong Chinese; Immunoblotting, dot

Introduction

The *ras* proto-oncogene encodes a guanine nucleotide-binding protein which is involved in signal transduction.[1] There are three members of the *ras* family, namely, H-, K- and N-*ras*. Despite being different in genetic structure and located on different chromosomes, the gene products have similar functions. Activation of *ras* genes due to mutations has been shown to result in cellular transformation.[2]

Myelodysplastic syndrome (MDS) is a monoclonal stem cell disorder which results in uncoupled proliferation and maturation of the haematopoietic progenitors. The disease is clinically manifested by peripheral pancytopenia and a hypercellular bone marrow.[3] The incidence of *ras* mutation in MDS has been reported to be approximately 30%.[1] Mutation of N-*ras* is the predominant type involved and the hot spots for mutations are codons 12, 13 and 61.[4]

The definitive role of N-*ras* mutation in disease development is controversial. Most of the information obtained so far in *ras* mutation is based on studies performed in Western populations. We have undertaken an investigation of N-*ras* mutation in Hong Kong Chinese MDS patients. In this study, presentation bone marrow samples were screened for the presence of N-*ras* mutation by using the polymerase chain reaction (PCR)/dot blot hybridization technique. Follow up studies were performed in some cases.

Materials and methods

Patient samples

Bone marrow specimens were obtained from 20 patients with MDS. According to the French-American-British (FAB) classification,[5] there were six cases of refractory anaemia (RA), seven cases of refractory anaemia with excess of blasts (RAEB), five cases of refractory anasemia with excess of blasts in transformation (RAEB-T) and two cases of chronic myelomonocytic leukaemia (CMML). Patient DNA was extracted according to standard procedures[6] from the bone marrow mononuclear cells which were separated using Ficoll-Hypaque (Pharmacia, Uppsala,

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Sweden). In seven patients, follow up samples were available.

Normal bone marrow samples
Normal bone marrow samples were obtained from 10 healthy bone marrow donors (with informed consent) as part of the bone marrow transplantation programme.

Control cell lines
Three cell lines were used as positive controls in the dot-blot hybridization system. They were the human myelogenous leukaemia cell line, HL-60, which has substitution of leucine for glutamine at codon 61,[7] the human acute T-lymphoblastic leukaemia cell line, MOLT-4, which has substitution of cysteine for glycine at codon 12,[8] and the human fibrosarcoma cell line, HT1080, which has substitution of lysine for glutamine at codon 61.[7]

Polymerase chain reaction amplification
Regions encompassing codons 12 and 13 in exon I, and codon 61 in exon II were amplified using the following primer pairs:[8]

codons 12 and 13	5'GACTGAGTACAAACTGGTGG
	3'CTCTATGGTGGGATCATATT
codon 61	5'GGTGAAACCTGTTTGTTGGA
	3'ATACACAGAGGAAGCCTTCG

These were 20-base synthetic oligonucleotides which were synthesised locally (institute of Molecular Biology, University of Hong Kong, Hong Kong).

The PCR was set up according to a protocol modified from Bashey et al.[9] The PCR mixture contained a final concentration of 1 x Taq polymerase buffer (Promega Corporation, Madison, Wi, US), 2.5 mmol/L magnesium chloride, 50 μmol/L of each dNTP (Promega Corporation, Madison, Wi, US), 25 μmol of each of the four primers, 1 unit Taq polymerase (Promega Corporation, Madison, Wi, US) and 1 μg genomic DNA. Double deionized water was added to make the final volume up to 50 μL. A 30-cycle reaction was run in a Programmable Thermal Controller (MJ Research Inc., Watertown, Ma, US). Each cycle consisted of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute.

Dot blot hybridization
Two microlitres of the amplified product were denatured and diluted in 1 volume of 20 x standard saline citrate (SSC), which was then spotted onto Hybond N Plus nylon

Table 1. List of oligonucleotide probes used for N-*ras* mutation screening

Oligomer	Sequence								Amino acid
gly_{12}	GGA	GCA	GGT	GGT	GGT	GTT	GGG	AA	glycine (wild type)
ser_{12}			AGT						serine
cys_{12}			TGT						cysteine
arg_{12}			CGT						arginine
asp_{12}			GAT						aspartic acid
ala_{12}			GCT						alanine
val_{12}			GTT						valine
gly_{13}	GGA	GCA	GGT	GGT	GTT		GGG	AA	glycine (wild type)
ser_{13}				AGT					serine
cys_{13}				TGT					cysteine
arg_{13}				CGT					arginine
asp_{13}				GAT					aspartate
ala_{13}				GCT					alanine
val_{13}				GGT					valine
gln_{61}	ACA	GCT	GGA	CAA	GAA		GAG	TA	glutamine (wild type)
glu_{61}				GAA					glutamic acid
lys_{61}				AAA					lysine
arg_{61}				CGA					arginine
leu_{61}				CTA					leucine
pro_{61}				CCA					proline
$his1_{61}$				CAT					histidine 1
$his2_{61}$				CAC					histidine 2

membrane (Amersham International, Bucks, UK) under vacuum using a Bio-dot apparatus (Bio-Rad, Hercules, Ca, US). The membrane was soaked in 0.4 mol/L NaOH for 20 minutes and was rinsed in 5 x sodium sodium phosphate ethylenediaminetetraacetic acid (SSPE) for 1 minute. The membrane was prehybridized at 56°C overnight in the following mixture (final concentration) 5 x SSPE, 5 x Denhardt's solution, 0.05 mol/L Tris-HCl (pH 8.0), 100 mol/L salmon sperm DNA, 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulphate (SDS). A segment of each synthetic oligomer (100 to 500 ng) was 5' end-labelled by incubation with 5 μL of labelled ATP (γ^{32}P) [Amersham International, Bucks, UK] and 1 μL of T4 kinase (Promega Corp., Madison, Wi, US), in the manufacturer's buffer at 37°C for 20 minutes. End-labelled oligomer was purified from unincorporated ATP by ethanol precipitation.[10] The γ^{32}P-labelled oligomer probe was added to the hybridization mixture at a concentration of 10^8 cpm/L. Following hybridization at 56°C for four hours, the membrane was rinsed for 10 minutes in 2 x SSPE with 0.1% SDS at room temperature. The membrane was then washed for 30 minutes in hybridization buffer without Denhardt's solution and salmon sperm DNA. Washing

Table 2. Clinical characteristics and N-*ras* mutation results of MDS patients

Patient	FAB subtype	Specimen date (m/y)	Blast (%)	N-*ras*
1a	RAEB	2/92	12	wt
1b	Post-BMT (remission)	3/92	0.6	wt
1c	RAEB-T	6/92	23	wt
2	RAEB-T	3/92	30	wt
3	CMML	4/92	4 (14)*	wt
4	RAEB-T	5/92	27	gln_{61}/arg_{61}
5a	RAEB	5/92	6	wt
5b	AM_6L	12/92	13	wt
6	RA	5/92	0	wt
		6/92	0	wt
7	RAEB	6/92	13	gln_{61}/arg_{61}
8	RAEB	7/92	6	wt
9	RAEB-T	7/92	28	wt
10a	RAEB	7/92	7	wt
10b	RAEB	10/92	not done	wt
10c	RAEB	1/93	not done	wt
11a	CMML	8/92	8 (12)	wt
11b	CMML	8/92	1 (20)	wt
12	RA	9/92	5	wt
13	RA	9/92	2	wt
14a	RA	5/92	1	wt
14b	RA	11/92	not done	wt
15	RAEB-T	10/92	19	wt
16a	RAEB	11/92	7	gly_{12}/ser_{12}
16b	RAEB	1/93	6	gly_{12}/ser_{12}
17	RAEB	12/92	7.5	wt
18	RA	12/92	2	wt
19	RA	1/93	2	wt
20a	RAEB-T	3/93	30	wt
20b	remission	5/93	5	wt

*Figures in brackets represent monocyte percentage in peripheral blood; wt = wild type

was carried out at 63°C for codons 12 and 13, or 59°C for codon 61.[8,11] The membrane was exposed to Kodak X-OMAT AR X-ray film (Kodak, Rochester, NY, US) at -70°C for 18 to 24 hours.

Probes used for hybridization

Twenty-one synthetic oligomers were used (Table 1), of which two were specific to wild-type alleles and the remaining 19 were specific to the possible mutations within codons 12, 13 and 61 of N-*ras*.[12]

Results

All patient samples gave positive hybridization signals with the wild-type probes indicating the presence of normal N-*ras* alleles. N-*ras* mutation was not detected in the 10 normal bone marrow samples examined, with the exception of one normal bone marrow, in which a faint dot was observed when probed with the arg_{61} probe (Fig 1: middle panel, C3). This weak signal could have resulted from inadequate washing of the hybridized membrane. It could also have been due to lowering of the washing temperature, which decreased the stringency of washing.

Three of the 20 MDS patients harboured mutant N-*ras* alleles (Table 2). Patient 4 (RAEB-T) and patient 7 (RAEB) had a glutamine for arginine substitution at codon 61 (Fig 1: middle panel, B2 and B5, respectively). A very weak hybridization signal was observed in patient 4 when probed with the leu_{61} probe (Fig 1: lower panel, B2), which again, may have been

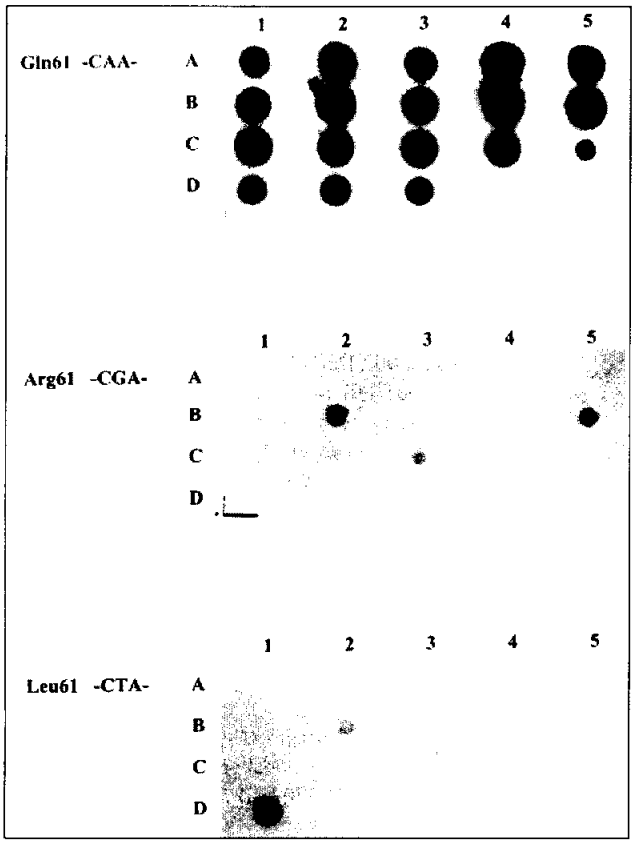

Fig 1. Detection of N-*ras* mutation at codon 61 in MDS patients. DNA samples from MDS patients (rows A, B), healthy individuals (row C), control cell lines HL-60 (D1), MOLT-4 (D2) and HT1080 (D3) were hybridized with wild-type probe (Upper panel), arg_{61} probe (Middle panel) and leu_{61} probe (Lower panel).

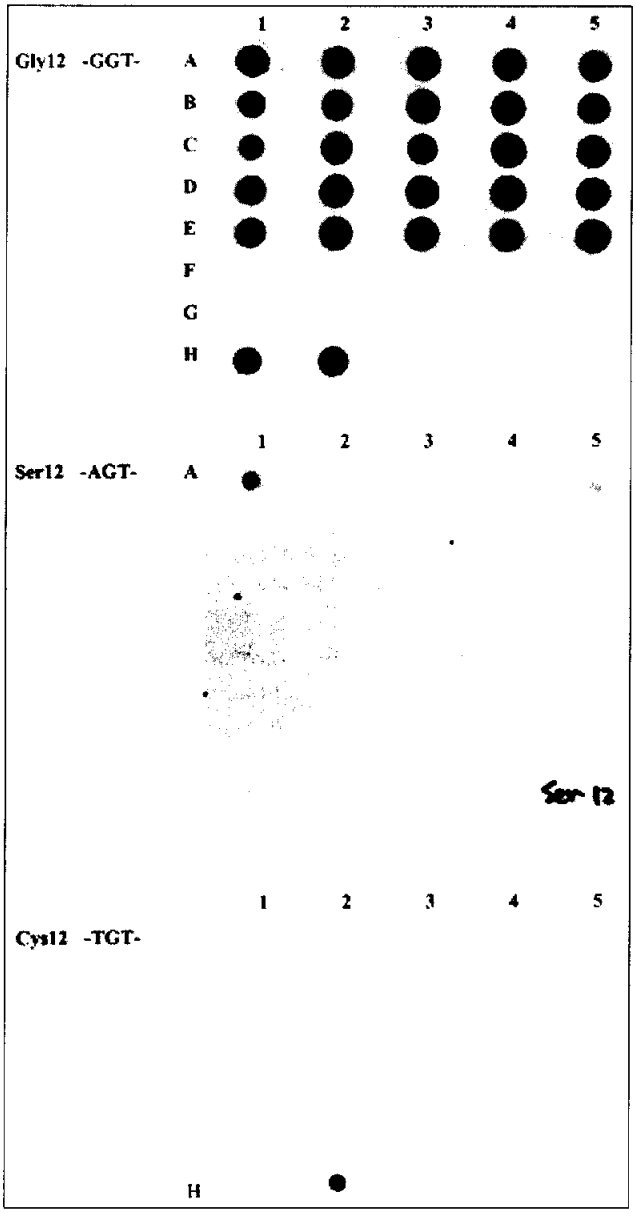

Fig 2. Detection of N-*ras* mutation at codon 12 in MDS patients. DNA samples from MDS patients (rows A-D), healthy individuals (row E), control cell lines HL-60 (H1) and MOLT-4 (H2) were hybridized with wild-type probe (Upper panel), ser_{12} probe (Middle panel) and cys_{12} probe (Lower panel).

due to insufficient washing of the membrane. In patient 16 (RAEB) no mutant N-*ras* was detected in the presentation bone marrow screening (Fig 2: middle panel, A5). However, in the follow up bone marrow sample, a glycine for serine substitution at codon 12 was detected (Fig 2: middle panel, A1).

In six patients (1, 5, 10, 11, 14 and 20) both diagnostic and follow up samples were studied but only wild-type alleles of N-*ras* were found (Table 2). This included patient 1 when an analysis was performed in relapse, three months after bone marrow transplantation, and in patient 5 when the disease had progressed to acute myeloid leukaemia (AML) M_6 subtype, six months after diagnosis.

Discussion

The incidence of N-*ras* mutation in Hong Kong Chinese MDS patients was found to be 15% (3 of 20 cases) by the PCR/dot-blot hybridization procedure. This result was within the range of incidence (3% to 33%) reported by various investigators in previous studies.[13] The variation in results might be due to the difference in techniques employed, the inadequacy of sample sizes studied by different investigators, and the heterogeneous nature of the disease.

It has been demonstrated the N-*ras* mutation in myeloid leukaemia may be associated with previous therapy with alkylating agents[1] for treatment of solid tumours, i.e. N-*ras* mutation may arise as a secondary event. However, in this study, none of the patients with mutant N-*ras* has received previous chemotherapy when the presentation bone marrow was taken.

Previous studies showed that N-*ras* mutation can be found in both early and late disease states.[14] In our study, all three N-*ras* mutations were found in the more progressive RAEB and RAEB-T subtypes. This finding is consistent with that of Nakagawa et al, who found three of four MDS cases with N-*ras* mutation belonging to the RAEB and RAEB-T subtypes.[15] However, N-*ras* mutation was not found in patient 1 when he relapsed three months after a bone marrow transplant or in patient 5 when this patient was transfused from RAEB-T to AML M_6 subtype. Whether or not N-*ras* mutation is associated with disease prognosis could be confirmed by carrying out longitudinal studies of *ras* mutation in a larger series of patients with MDS.

In patient 16, mutant N-*ras* was detected in the follow up bone marrow sample (Fig 2: middle panel, A1). However, there was no change in the bone marrow blast percentage compared with that found at diagnosis. The significance of this finding is uncertain. However, the finding is in keeping with the proposition that the N-*ras* gene mutation may occur with progression of disease in myelodysplastic syndromes.

Acknowledgements

We thank Mr C So for excellent technical advice and Dr E Chiu for supply of normal bone marrow samples. Ms P Leung was supported by an M Phil studentship and a Commonwealth Research Committee grant.

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