ANALYSIS OF *RAS* GENE MUTATIONS IN ACUTE MYELOID LEUKEMIA BY THE POLYMERASE CHAIN REACTION AND OLIGONUCLEOTIDE PROBES

Y M Chin, J J Bosco, C L Koh

ABSTRACT

In vitro deoxyribonucleic acid (DNA) amplification by the polymerase chain reaction (PCR) followed by hybridization with oligonucleotide probes were used to study ras gene mutations in acute myeloid leukemia (AML). The DNA of 30 AML patients at presentation of the disease at the University of Malaya Hospital, Kuala Lumpur were screened for ras gene mutations in codons 12, 13 and 61 of the N-ras, K-ras and H-ras genes. Four patients (13.3%) had ras gene mutations. They were all below their early thirties in age. Of the four patients with ras gene mutations, three were M3 and one was M4 according to the French American British (FAB) classification of AML.

Keywords : Ras gene mutations, Acute myeloid leukemia

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INTRODUCTION

The human *ras* gene consists of three members : the H-*ras*, K-*ras* and N-*ras*. Point mutations which activate the ras proto-oncogenes to oncogenes with transforming potential may be one of the mechanisms in the development of malignancy. These point mutations which result in the substitution of the normal amino acid by a different amino acid usually occur in condons 12, 13 and 61 of the H-*ras*, K-*ras* and N-*ras* genes. The frequency of *ras* gene mutations vary with the type of leukemia, 36% in AML, 9% in acute lymphoblastic leukemia (ALL) and 5% in chronic phase of chronic myeloid leukemia (CML). About 90% of *ras* gene mutations in human leukemias are found in the N-ras genes [1].

The three human *ras* genes encode homologous proteins of 21 kilodalton (kd) in molecular weight. The 21 kd *ras* protein is located at the inner surface of the plasma membrane. The *ras* proteins can bind guanosine nucleotide diphosphate (GDP) and guanosine nucleotide triphosphate (GTP) with high affinity and also possess guanosine nucleotide triphosphatase (GTPase) activity[2]. The *ras* proteins resemble the 'G proteins' and are known to be involved in the transduction of receptor-mediated external signals into the cell[3].

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The current hypothesis is that the normal *ras* protein bind GTP in response to some receptor-mediated signal and in doing so transduce the signal further into the cell. Signal transmission would terminate with the GTPase activity converting GTP to GDP.

Ras gene mutations may result in the mutated *ras* proteins having reduced GTPase activity[4] and leading to prolonged binding of GTP and hence prolonged activation. In this way the proper functioning of the signal pathway involving the *ras* proteins would be disturbed. Since *ras* proteins are involved in the control of the cell cycle, these changes may contribute significantly towards uncontrolled growth, as seen in tumour cells.

A single *ras* gene mutation is not sufficient to cause malignant transformation. The cooperation of other oncogenes or a second genetic event is required[5].

In the present study for the detection of *ras* gene mutations in AML the PCR was used to amplify specific regions of the DNA to about more than a million fold after 30 cycles of *in vitro* DNA synthesis. The amplified DNA was probed with different oligonucleotide probes to detect the various type of point mutations.

MATERIALS AND METHODS

Patients

DNA was isolated from the peripheral blood or bone marrow of 30 AML patients at presentation of the disease.

In vitro DNA amplification

About one to three μg of genomic DNA was subjected to 30 cycles of PCR amplification with a pair of *ras* amplimers in an automated heat block (DNA thermal cycler, Perkin-Elmer Cetus, USA) in the presence of a thermostable enzyme, *Taq* DNA polymerase. Each cycle consists of 3 steps : denaturation at 95°C for 2 minutes, annealing at 50°C for 2 minutes and synthesis at 60°C for 2 minutes. A total of 6 pairs of *ras* amplimers, each amplimer about 20 bases in length (Clontech, USA) were used. The amplified DNA was slotted onto a nylon filter under vacuum with a Bio-Dot apparatus (Bio-rad, USA) and fixed onto the nylon filter by ultra violet light illumination. The filter was prehybridized for an hour at 37°C in PCR hybridization mixture.

Oligonucleotide probe hybridization

For the detection of *ras* oncogene point mutations a total of 66 oligonucleotide probes with different types of point mutations at codons 12, 13 and 61 of the H-*ras*, K-*ras* and N-*ras* genes (Clontech, USA) were end labelled with γ-^{32}p Adenosine triphosphate (ATP) specific activity 3,000 Ci/mmol) using T4

Table I - Data of AML Patients with *Ras* Gene Mutations

No.	Patient	Age At Onset	Sex	Ethnic Group	Specimen	Peripheral Blood WBC x 10^9 litre	Peripheral Blood % Blasts	FAB Classification	Gene	Wild Type Codon	Wild Type Amino Acid	Mutant Codon	Mutant Amino Acid
1	MTH	7	M	Chinese	PB	20.7	Predominantly Promyelocytes	AML (M3)	N-ras 12	GGT	Glycine	AGT	Serine
2	LKT	32	M	Chinese	BM	27.0	9	AML (M3)	K-ras 12	GGT	Glycine	GAT	Aspartic Acid
3	MNA	7	M	Malay	BM	148.4	5	AML (M3)	N-ras 12	GGT	Glycine	GTT	Valine
4	LNY	23	C	Chinese	BM	105.0	6	AML (M4)	N-ras 61	CAA	Glutamine	CTA	Leucine

LEGEND:

Sex: M = Male, F = Female

Specimen: BM = Bone Marrow, PB = Peripheral Blood

polynuncleotide kinase. The labelled probes were purified through a sephadex G-50 column. At least two labelled probes were added simultaneously to the prehybridized nylon filters and the hybridization was carried out overnight at 37°C. The filters were subjected to high stringency wash in 3M tetramethylammonium chloride (TMAC) wash solution at 61°C. The filters were then autoradiographed at -70°C using X-ray films with intensifying screens for about 30 minutes to 2 hours.

The nylon filters were then deprobed in deprobing solution at 75°C for 30 minutes. The filters were reprobed again with another set of labelled probes.

RESULTS

Four out of 30 AML patients (13.3%) at presentation of the disease were found to have a *ras* gene mutation. There were 13 males and 17 females. The age of the 30 AML patients ranged from 2 to 74 years with a median age of 35 years. All the four patients with *ras* gene mutations were below their early thirties in age.

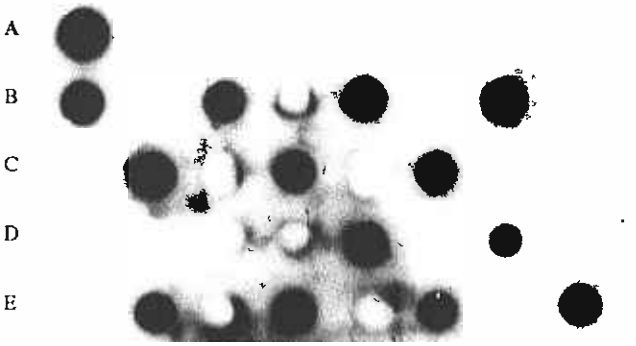
The biological and haematological data as well as the type of *ras* gene mutation present in all the 4 patients are summarised in Table I. The FAB classification of the 30 AML patients are as follows: M1 six patients, M2 one patient, M3 eight patients, M4 twelve patients and M5 three patients. Of the four patients with *ras* gene mutations, three were M3 and one was M4. N-*ras* gene mutations were detected in 3 patients while K-*ras* gene mutation was found in one patient only.

Dot blot analysis and autoradiographs of some AML DNA samples screened for N-*ras* 12 mutations are shown in Fig 1(a) - (c). Fig 1(a) shows the hybridization of the amplified AML DNA with the N-*ras* 12 wild type (normal) probe, codon GGT. Dot blot analysis of point mutations at N-*ras* 12 characterised by substitution of the normal amino acid glycine (GGT) by serine (AGT) in patient MTH and by valine (GTT) in patient MNA are shown in Fig 1(b) and Fig 1(c) respectively. Patient LNY had a mutation at N-*ras* 61 resulting in substitution of glutamine (CAA) by leucine (CTA) while the K-*ras* 12 mutation in patient LKT resulted in substitution of glycine (GGT) by aspartic acid (GAT).

DISCUSSION

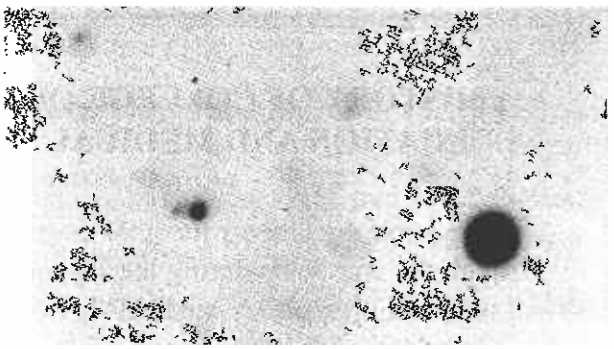
Padua et al, 1988[6] found that myelodysplastic syndrome (MDS) patients with *ras* gene mutations evolve more frequently to AML than those without. Their findings were however contradicted by Bar-Eli et al, 1989[7]. N-*ras* gene mutations in childhood ALL tend to correlate with a poor prognosis with a higher risk for haematologic relapse and a trend towards lower rate of complete remission [8].

Fig.1 - Dot Blot Analysis & Autoradiographs of AML patients Hybridization of the amplified DNA with the wild type (normal) N-*ras* 12 probe (codon GGT which codes for glycine). The same nylon filter was deprobed and reprobed with N-*ras* 12 probes with different types of point mutations.



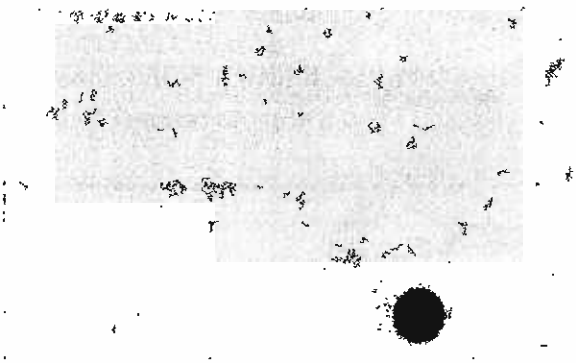
N 12 GLYCINE – GGT

(b) Point mutation in patient MTH detected by mutant N-*ras* 12 probe, codon AGT (serine). The mutation results in substitution of glycine by serine.



N 21 SERINE – AGT –

(c) Point mutation in patient MNA detected by mutant N-*ras* 12 probe, codon GTT (valine). The mutation is characterised by substitution of glycine by valine.



N 12 VALINE –GTT–

Studies from western countries[9,10] showed no specific association of *ras* gene mutations with patients' age, sex, clinical course, karyotype and immunophenotype. *Ras* gene mutations occur more frequently in M4 than in M1, M2 or M5. *Ras* gene mutations can be present at various stages during the development of AML. Double *ras* gene mutations are not uncommon. When patients achieve remission after therapy the *ras* mutations at initial presentation was undetectable, suggesting that these mutations are acquired. At relapse the same mutation may or may not be present. A mutation not present at presentation may appear at relapse also. The presence of *ras* gene mutations in AML at diagnosis but not at relapse suggest that *ras* gene mutations may be important for leukemia development but may not be necessary to maintain the transformed phenotype.

The prognostic significance of *ras* gene mutations and its increased expression in AML is still unclear. In the present study, all the four AML patients with *ras* gene mutations are below their early thirties in age. *Ras* mutations occur more frequently in the M3 subtype than in other subtype of AML. Further research is required regarding the prognostic significance of *ras* gene mutations and also our findings in the Malaysian AML patients because of the small number of patients with *ras* gene mutations.

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