Haematology and Blood Transfusion Vol. 29
Modern Trends in Human Leukemia VI
Edited by Neth, Gallo, Greaves, Janka

Somatic N-*ras* Oncogene Activation in a Patient with Acute Myeloblastic Leukemia *

C. Moroni, C. Gambke, E. Signer, and J. Jiricny

A. Introduction

The recognition that retroviral oncogenes (v-*onc*) are derived from normal cellular sequences termed proto-oncogenes or c-*onc,* has led to a search to see whether human proto-oncogenes present in human tumors show alterations in structure or expression. Of particular interest are the activated forms of *ras* proto-oncogenes; these can be assayed in a biologic test system, since activated *ras* genes from tumor tissue, but not normal alleles have the ability to transform NIH 3T3 cells in tissue culture [1–4]. The role, if any, of this transforming activity in the natural history of the human tumor is still unknown. The human genome contains three functional *ras* genes, localized on different chromosomes: H-*ras* (related to v-*ras* of Harvey sarcoma virus), K-*ras* (related to v-*ras* of Kirsten sarcoma virus), and N-*ras,* a *ras* family member identified by nucleic acid hybridization through its relatedness to the former *ras* genes [5–8]. Activation of *ras* genes was found to be the result of point mutations altering amino acid 12 of the H-*ras* or K-*ras* [11–18] or amino acid 61 of the H-*ras* or N-*ras* genes [9, 10, 19].

We have initiated a study to see whether human leukemias, prior to treatment, contain activated *ras*-genes, and if so, whether and how the presence of the activated gene correlates with the course of the disease. In this report, we summarize our analysis of a patient with acute myeloblastic leukemia (AML) with an activated N-*ras* gene.

B. Materials and Methods

I. NIH 3T3 Transfection Test

High molecular weight DNA was isolated by phenol and subsequent chloroform extraction as described [20]. DNA was precipitated by the calcium phosphate method on NIH 3T3 cells, seeded one day before at 5×10^5 cells per 10-cm plate, and foci of transformed cells were enumerated after 2 weeks. Details of the procedure are given in [20].

II. Oligonucleotide Synthesis

The oligonucleotides were synthesized by the modified phosphotriester approach described by Sproat and Bannwarth [21], using a semiautomated continuous-flow benchtop synthesizer of our own design. The synthesis were carried out starting with 40 mg controlled-pore glass support, corresponding to approximately 4 μmol nucleoside functionality; 40 mg mononucleotide building blocks and 60 mg condensing agent (mesitylenesulfonylnitrotriazolide, MSNT) were used for each addition. The cycle time was 24 min.

The products were fully deprotected and purified by ion exchange HPLC, using a Partisil 10/SAX 25 analytic column, eluted with a linear gradient of 0.001–0.4 *M*

* Friedrich Miescher-Institut, P.O. Box 2543, CH-4002 Basel, and Children's Hospital, University of Basel, Basel, Switzerland

potassium phosphate buffer pH 6.5, containing 60% formamide. Desalting was achieved by means of a Biogel P2 column (2.5 × 50 cm), eluted with a mixture of ethanol and water (2:8 v/v). The pure oligonucleotides were 5′-labeled with ^{32}P following the method of Smith and Zoller [22].

III. Southern Blot Analysis of Cloned N-*ras* Fragments

Plasmids containing the transforming, "activated gene", and the nontransforming, "normal gene", N-*ras,* respectively, were electrophoresed on 0.8% agarose gels. After transfer of DNA to nitrocellulose, hybridization was done in the presence of 10% dextran sulfate for 18 h at 40 °C using 1.4×10^6 cpm/ml labeled "normal probe" (see Fig. 2a) or 0.8×10^6 cpm/ml "activated probe" (see Fig. 2b). Filters were washed 3 × 15 min at 8 °C with 2 × SSC, 3 min at 40 °C with 2 × SSC in 0.1% SDS, and 4 min at 54 °C with 2 × SSC in 0.1% SDS, and autoradiographed for 6 days at − 70 °C.

C. Results and Discussion

We have recently described a patient with AML where a NIH 3T3 transfection test performed with bone marrow-derived DNA was positive [20]. The salient laboratory data of this patient together with transfection data are summarized in Table 1. The focus-forming activity of this DNA (0.056 foci per microgram DNA), which is derived from a marrow with 68% atypical blasts is comparable to values obtained by other workers with DNA from cloned cell lines. This suggests that the transforming gene may be represented clonally in all atypical myeloblasts and thus might have been present early in the history of this malignant clone, perhaps at the time of the leukemogenic transformation. The transforming gene was not present in the germ line of this patient, as DNA from cultured fibroblast cells did not have transforming activity. Thus, the generation of the transforming gene must have been a somatic

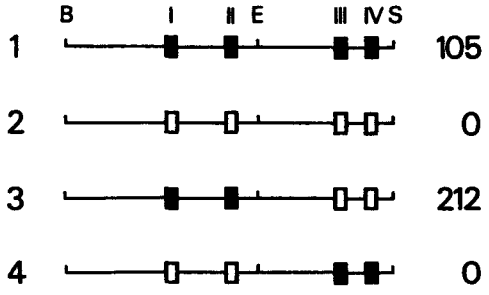

Fig. 1. Transforming activity of chimeric N-*ras* molecules. The four exons of the N-*ras* gene reside on a 12.5 kilobases Bam HI-Sac I fragment [10]. An Eco RI site separates exons I and II from exons III and IV. From cloned normal and transforming N-*ras* genes, Bam HI-Eco RI and Eco RI-Sac I fragments were isolated, ligated to construct chimeric molecules (3, 4) and to reconstruct transforming (1) and normal (2) molecules for control. The ligation mixture was assayed on NIH 3T3 cells for transforming activity. The numbers on the right indicate the number of foci obtained after 2 weeks. *Full boxes* exons of transforming N-*ras* allele; *open boxes* exons of normal N-*ras* allele; B Bam HI; E Eco RI; S Sac I

Table 1. Hematologic data and transforming activity of AML bone marrow DNA[a]

NIH 3T3 transfection test	Bone marrow DNA: 0.056 foci/μg DNA Fibroblast DNA: 0.002 foci/μg DNA
Bone marrow	Hypercellular 68% Atypical myeloblasts (peroxidase +ve, Sudan black +ve, PAS − ve, nonspecific esterase − ve) Karyotype: no abnormal findings
Blood	WBC: $62 \times 10^9\ l^{-1}$ (mostly atypical myeloblasts) Platelets: $55 \times 10^9\ l^{-1}$ Hemoglobin: 91 g l^{-1}

[a] At time of diagnosis and before chemotherapy administration

Table 2. Mutations affecting amino acid 12 of the *ras* genes

Gene	Codon	Amino acid	Origin	References
N-*ras*	GGT	Gly	Normal DNA	[9, 10]
	GAT	ASP	AML	This study; [23]
H-*ras*	GGC	Gly	Normal DNA	[11]
	GTC	Val	Bladder carcinoma EJ, T24	[11, 12, 13, 14]
K-*ras*	GGT	Gly	Normal DNA	[14]
	TGT	Cys	Lung carcinoma Calu-1	[15, 16]
	TGT	Cys	Lung carcinoma PR371	[17]
	GTT	Val	Colon carcinoma SW480	[16]
	CGT	Arg	Bladder carcinoma A1698	[18]
	CGT	Arg	Lung carcinoma A2182	[18]
	CGT	Arg	Lung carcinoma LC-10	[18]

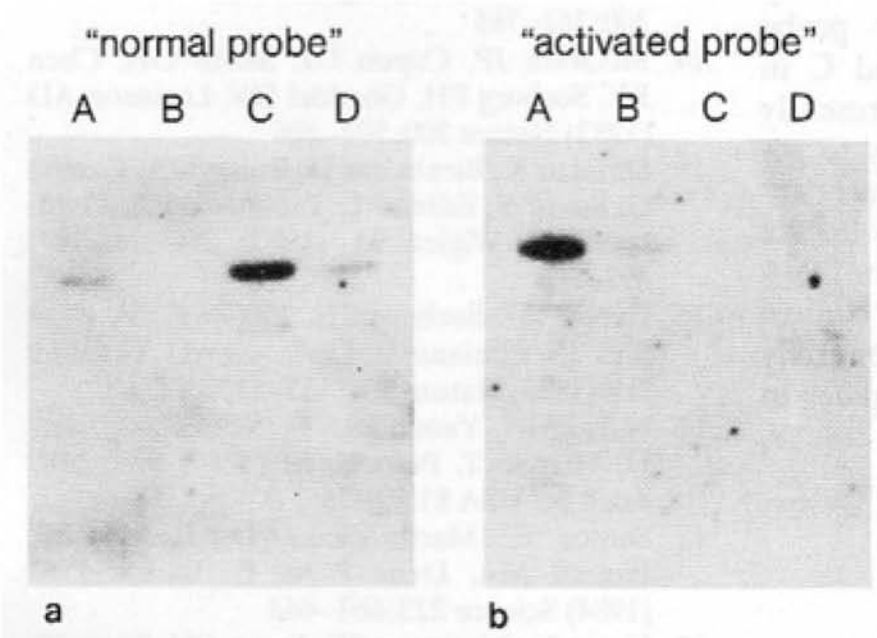

Fig. 2a, b. Southern blot analysis of cloned transforming, "activated gene" and nontransforming "normal gene" N-*ras* genes using synthetic ^{32}P-labeled heptadecanucleotides as probes. **a** "normal probe" complementary to normal N-*ras* gene, **b** "activated probe" complementary to mutated N-*ras* gene. The sequence of the normal probe is TGGAGCAGGTGGTGTTG. The sequence of the activated probe is TGGAGCAGATGGTGGTG. The triplet coding for amino acid 12 is *underlined.* A activated gene 1.0 ng; B activated gene 0.1 ng; C normal gene 1.0 ng; D normal gene 0.1 ng

event. This gene was identified as N-*ras* by analyzing primary and secondary transfection foci [20]. Both the transforming N-*ras* gene (derived from a secondary locus) and the nontransforming N-*ras* gene (derived from the patient's fibroblasts) were cloned in phage *L47.1* as described elsewhere [23]. The four exons of the N-*ras* gene are localized on a Bam HI-Sac I fragment (Fig. 1), where an Eco RI site separates exons I and II from exons III and IV. The chimeric N-*ras* molecules between the transforming and nontransforming gene fragments were constructed from subfragments (Fig. 1), and analyzed for transforming activity. As shown in Fig. 1, focus formation was only seen when exons I/II from the transforming gene were present, which suggested that a mutation occurred in exons I/II. These exons were therefore sequenced. While the sequence of the nontransforming N-*ras* gene was identical to the published N-*ras* sequence [9, 10], the transforming gene differed in one nucleotide. The GGT triplet coding for amino acid 12 was altered to GAT which changes the coding from Gly to Asp. The two formerly known N-*ras* activations seen in a neuroblastoma line and a fibrosarcoma line both affect amino acid 61 [9, 10]. However, as already mentioned, alterations at positions 12 have been observed with H-*ras* and K-*ras* genes. Table 2 summarizes the codon 12 nucleotide changes of all *ras* genes observed so far. Both the first and second G can be altered, 7/8 cases involve G → T or

G → C transversions, while our case involves a G → A transition.

Since, in all appropriately analyzed cases of *ras* activation, one finds an alteration of amino acid 12 or 61, one may, using suitable oligonucleotide probes, diagnose a mutational event by Southern blot analysis. This would circumvent the time-consuming and cumbersome NIH 3T3 transfection test. To approach this possibility we have synthetized two heptadecanucleotides corresponding to the normal and the mutated N-*ras* gene (Fig. 2). In preliminary experiments using cloned normal and mutated N-*ras* genes, we found hybridization conditions under which each probe specifically hybridized with its homologous allele. Thus, a G → A transition can be positively or negatively detected by either probe (compare for example lanes A and C in both examples shown). We are presently trying to establish this methodology to genomic DNA. It should be particularly interesting to use these probes to assay for the presence of the N-*ras* mutation in DNA isolated from bone marrow during clinical remission. Such probes should be generally useful for studying the role of *ras* genes in leukemias and other malignancies.

References

1. Parada LF, Tabin CJ, Shih C, Weinberg RA (1982) Nature 297:474–478
2. Der CJ, Krontiris TG, Cooper GM (1982) Proc Natl Acad Sci USA 79:3637–3640
3. Pulciani S et al. (1982) Nature 300:539–541
4. Santos E, Tronick SR, Aaronson SA, Pulciani S, Barbacid M (1982) Nature 298:343–347
5. Ellis RW, DeFeo D, Shih TY, Gonda MA, Young HA, Tsuchida N, Lowy DR, Scolnick EM (1981) Nature 292:506–511
6. Taparowski E, Shimizu K, Goldfarb M, Wigler M (1983) Cell 34:581–586
7. Marshall CJ, Hall A, Weiss RA (1982) Nature 299:171–173
8. Murray MJ, Cunningham JM, Parada LF, Dautry F, Lebowitz P, Weinberg RA (1983) Cell 33:749–757
9. Wigler M, Pellicer A, Silverstein S, Axel R, Urlab G, Chasin L (1979) Proc Natl Acad Sci USA
10. Brown R, Marshall CJ, Pennie SG, Hall A (1984) EMBO J 3:1321–1326
11. Tabin CJ, Bradley SM, Bargmann CI, Weinberg RA, Papageorge AG, Scolnick EM, Dhar R, Lowy DR, Chang EH (1982) Nature 300:143–149
12. Reddy EP, Reynold RK, Santos E, Barbacid M (1982) Nature 300:149–152
13. Taparowski E, Suard Y, Fasano O, Shimizu K, Goldfarb M, Wigler M (1982) Nature 300:762–765
14. McGrath JP, Capon DJ, Smith DH, Chen EY, Seeburg PH, Goeddel DV, Levinson AD (1983) Nature 300:501–506
15. Shimizu K, Birnbaum D, Ruley MA, Fasano O, Suard Y, Edlund L, Taparowsky E, Goldfarb M, Wigler M (1983) Nature 304: 497–500
16. Capon DJ, Seeburg PH, McGrath JP, Hayflick JS, Edmann U, Levinson AD, Goeddel DV (1983) Nature 304:507–513
17. Nakano H, Yamamoto F, Neville C, Evans D, Mizuno T, Perucho M (1984) Proc Natl Acad Sci USA 81:71–75
18. Santos E, Martin-Zanca D, Reddy EP, Pierotti MA, Della Porta G, Barbacid M (1984) Science 223:661–664
19. Yuasa Y, Srivastava SK, Dunn CY, Rhim JS, Reddy EP, Aaronson SA (1983) Nature 303: 775–779
20. Gambke C, Signer E, Moroni C (1984) Nature 307:476–478
21. Sproat BS, Bannwarth W (1983) Tetrahedron Letters 24:5771–5774
22. Zoller MJ, Smith M (1983) Methods Enzymol 100:468–500
23. Gambke C, Hall A, Moroni Ch (1984) Proc Natl Acad Sci USA 82:879–882