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Ras gene mutations in patients with acute myeloid leukaemia and exposure to chemical agents

Emanuela Barletta[1], Giuseppe Gorini[2], Paolo Vineis[3], Lucia Miligi[2], Laura Davico[4], Gabriele Mugnai[1], Stefania Ciolli[5], Franco Leoni[5], Marilena Bertini[4], Giuseppe Matullo[6] and Adele Seniori Costantini[2,7]

[1]Department of Experimental Pathology and Oncology, University of Florence, [2]Centre for Study and Prevention of Cancer—Unit of Occupational and Environmental Epidemiology, Florence, [3]Department of Biomedical Sciences and Human Oncology, AO San Giovanni Battista/University of Turin, [4]Department of Haematology, AO San Giovanni Battista, Turin, [5]Department of Haematology Careggi University Hospital, Florence and [6]ISI Foundation and Department of Genetics, Biology and Biochemistry, University of Turin, Turin, Italy

[7]To whom correspondence should be addressed
Email: a.seniori@cspo.it

Mutations of the N- and K-*ras* genes occur in ~15–30% of acute myeloid leukaemia patients. The role of the oncogenic *ras* in leukaemogenesis remains unclear. Few studies have revealed that mutations in the *ras* oncogene family are more probably found in acute myeloid leukaemia patients with previous exposure to toxic agents. A case–case study was conducted in the areas of Florence and Turin, Italy, to investigate whether the presence of N- and K-*ras* mutations in acute myeloid leukaemia patients was related to a higher frequency of exposure to chemicals. During a 3-year period, 111 acute myeloid leukaemia patients were enrolled. All the patients were interviewed using a semi-structured questionnaire collecting data on residential history, occupation, personal habits and pathological history. The presence of N- and K-*ras* mutations was analysed by amplification and synthetic oligonucleotide probes and by the so-called polymerase chain reaction amplification for specific alleles technique. A total of 34 (30.6%) patients were found to harbour *ras* mutations in N-*ras* and/or K-*ras*. Fourteen patients (12.6%) had a single *ras* mutation and 20 patients (18%) had two *ras* mutations. A positive association between a priori at risk jobs and *ras* mutations was found, based on nine exposed cases; the odds ratio, adjusted by age, sex and previous X-ray and/or chemotherapy was 2.8 (95% confidence intervals: 0.9–9.0). When considering only subjects with two *ras* mutations the odds ratio was 4.8 (95% confidence intervals: 1.2–18.8). The odds ratio for a previous X-ray and/or chemotherapy was 16.2 (95% confidence intervals: 1.8–755.9); when only subjects with two *ras* mutations were considered, the odds ratio was 26.1 (95% confidence intervals: 2.5–1248.9). In conclusion, our data suggest that *ras* oncogene mutations might identify a group of leukaemia in people with previous X-ray/chemotherapy or with exposure to chemical agents in the work environment.

Abbreviations: AML, acute myeloid leukaemia; GTP, guanosine triphosphate; JSQ, job-specific questionnaires; MDS, myelodysplastic syndrome.

Introduction

Acute myeloid leukaemia (AML) is a disease of the haematopoietic cell system in which there is an aberrant accumulation of immature myeloid cells in the peripheral blood and bone marrow. Activating and loss-of-function mutations are common in AML (1) and involve oncogenes, such as the *ras* super gene family, that play important roles in the process of proliferation and differentiation (2–4). Mutations of the N- and K-*ras* genes occur in ~15–30% of AML patients (5–7), while the background frequency of *ras* mutations in a normal population has yet to be determined; in one study by Taylor *et al*., *ras* mutations were identified in only two out of 115 normal individuals (8).

The role of the oncogenic *ras* in leukaemogenesis remains unclear (9,10). Results of animal studies suggest that mutation of *ras* family genes is an early event in chemically induced tumours (11,12). Some studies showed that, in human AML, not all leukaemia blasts contain mutated *ras* genes, and suggested that these mutations occurred during the progression of the disease (13); other studies showed a high frequency of *ras* mutations in pre-leukaemia conditions such as the myelodysplastic syndrome (MDS), and suggested that *ras* mutations might be an early event in the leukemogenesis process (14–17).

Data on *ras* mutations as possible biological markers of exposure to environmental agents in AML are limited. In a previous study by Taylor *et al*. (18) patients with *ras* mutation positive AML had a higher frequency of working for 5 years or more in a priori high-risk occupations than did patients with *ras*-negative AML. Furthermore, in this study, when *ras*-positive patients were compared with healthy controls, the odds ratio for occupational exposure remained elevated, whereas patients with *ras* mutation negative AML showed no increased risk when compared with control subjects. A more recent study examined *ras* mutations in a cohort of 63 people in the petrochemical industry, of which 44 were occupationally exposed to benzene. A K-*ras* mutation was found in one of 44 exposed workers (19). Another study by the same author showed a high frequency of N-*ras* mutations in children with secondary leukaemia after chemotherapy for a previous malignancy (20).

In the present study our aim has been to investigate whether the presence of N- and K-*ras* mutations in AML patients was related to a higher frequency of environmental exposures, particularly, exposures to solvents.

Material and methods

Study population enrolment and blood sample collection

Eligible cases included all AML patients, male and female, 20–74 years old, who were diagnosed using standard bone marrow and blood smears and immunophenotyping data as having AML according to the French–American–British classification (FAB), and who were resident in the Florence and Prato areas, or in the city of Turin. Cases were identified through the Department of Haematology—Policlinico Careggi—Florence, and the

Department of Haematology—Ospedale Maggiore, Turin, and were diagnosed between December 1, 1995, and December 1, 1998. Patients were asked to participate in the study and were invited to be interviewed by expert interviewers; patients were informed that their clinical, biological and anamnestic information would be used for research purpose.

Peripheral blood samples were obtained from the enrolled patients at the time of AML diagnosis. None of the patients had yet received therapy at the time of the blood draw. Genomic DNA was extracted from a leukocyte enriched fraction of whole blood (buffy coat) according to the QIAamp Blood Kit protocol (Qiagen GmbH, Hilden, Germany). DNA concentration was determined by measuring the absorbency at 260 nm and DNA purity was determined by calculating the ratio of absorbency at 260 nm to absorbency at 280 nm. Fifteen healthy donors (12 males and three females) provided blood samples as a control group.

ras gene amplification

Codons 12, 13 and 61 of the N- and K-*ras* genes were amplified by PCR according to the Human *ras* Onco-Lyzer Kits and Amplimer Sets protocols (Clontech, Palo Alto, CA). Briefly, 200 ng of genomic DNA were amplified for 30 cycles with 1.25 U of *Thermus aquaticus* DNA polymerase (Taq DNA Polymerase, Promega, Madison, WI) and 0.6 μM of each primer in a 50 μl final volume solution containing: 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatine, 0.2 mM of each of the four deoxynucleoside triphosphates. Each PCR cycle consisted of a denaturising step at 96°C for 1 min, annealing at 56°C for 1 min and extension at 74°C for 1 min. The last cycle was followed by a 7 min extension at 74°C. Aliquots (5 μl) of the amplification products (108–111 bop) were analysed by electrophoresis in 2% agarose gel. Primers used were from Clontech (Human N-*ras*/12,13, N-*ras*/61, Ki-*ras*/12,13 and Ki-*ras*/61 Amplimer Set).

Two types of negative controls were used: amplification reaction with no added DNA in every PCR experiment, and amplification reactions with DNA from normal individuals. Moreover, in order to introduce a codon 12 asp mutation in N-*ras* gene as a positive control, normal DNA was amplified by PCR using the following mismatched sense primer, which was designed with a substitution of an A for the normal G at position −2 from its 3′ end: 5′-ATG ACT GAG TAC AAA CTG GTG GTG GTT GGA GCA GAT-3′.

Detection of mutations

Two different techniques were used to detect point mutations of N- and K-*ras*: (i) allele-specific oligonucleotide hybridization following polymerase chain reaction amplification, which can identify a heterozygous mutant present in 10% (21,22); and (ii) in order to confirm results obtained with oligonucleotide hybridization, we used mutation-specific PCR amplification—also called '*PCR amplification for specific alleles*' (PASA)—because it is a more sensitive and specific method that allows detection of single point mutations in the presence of up to a 10^7-fold excess of normal DNA (22–25).

Following the first method, the PCR products were hybridized with oligonucleotide probes, which were specific for all the pertinent point mutations and wild-type sequence of each particular codon (Human *ras* Mutalyzer Probe Panel, Clontech). Briefly, a 20 μl aliquot of the PCR reaction mixture was denatured with 100 μl of a 0.4 M NaOH and 25 mM EDTA solution, heated at 95°C for 2 min, and neutralized on ice with 100 μl of 2 M Tris–HCl (pH 7.4). The entire solution was slot-blotted on nylon membrane (Hybond-N+, Amersham, Buckinghamshire, UK) using a blotting manifold (Bio-Dot SF Apparatus, Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Each specific oligonucleotide probe was labelled at the 3′-end with fluorescein-11-dUTP according to the ECL 3′-oligolabelling and Detection System protocol (Amersham). The filters were hybridized overnight at 42°C in the presence of each individual 3′-end labelled oligonucleotide probe and washed at 55°C for 30 min according to the ECL 3′-oligolabelling and Detection System protocol. The hybridized probes were then detected by incubating filters with an anti-fluorescein horseradish peroxidase-conjugated antibody and revealed according to the ECL detection protocol. Filters were exposed for 15 min to a Hyperfilm-ECL film (Amersham) for autoradiography. Films were then scanned in a grey-scale mode at 600 d.p.i. using the Astra 1220S scanner (Umax) equipped with a transparency option and interfaced to an IBM-compatible computer and slot-blotted bands were quantified by measuring the integrated density of their signal strength using the Scion Image for Windows software (Scion, Frederick, MD).

For PASA, mutation-specific sense primers were used, which contain a base substitution at their 3′-end corresponding to the N- and K-*ras* point mutations; the anti-sense primers were the same as those used to amplify codons 12, 13 and 61 of N- and K-*ras* genes. The PCR reaction (30 cycles, annealing temperature 56°C) was performed in a volume of 40 μl in a solution containing 50 mM KCl, 10 mM Tris–HCl at pH 8.3, 2 μM of each nucleotide, 0.7 mM of $MgCl_2$, 0.05 μM of each primer and 1 U of AmpliTaq DNA polymerase (Perkin Elmer Cetus, Milan, Italy). After PCR amplification, the products of the reaction were analysed by electrophoresis in 8% polyacrylamide gels. As TaqI polymerase enzyme lacks a 3′ exonuclease activity and is therefore unable to amplify DNA if the single base mismatch is located at the 3′-end of the primer, samples were scored positive for a specific point mutation if their PCR products developed in the reaction.

Collection of information and assessment of exposure to solvents

A semi-structured questionnaire, realized in the context of the 'Italian multicenter case-control study on hematolymphopoietic malignancies' (26), was used in order to collect data on residential and occupational history (productive sector and job), personal habits and medical history. Job-specific questionnaires (JSQ) were used to assess specific work tasks done by the subject. Jobs were classified according to the ILO classification (27). Criteria for defining exposure have been discussed widely (28). Two methods were utilized to define the subjects in the study that were exposed to solvents: (i) having ever worked in a job that could entail exposure to solvents; and (ii) having experienced exposure to solvents, as assessed, at individual level, by an expert industrial hygienist. Concerning the first method, all jobs held by the subjects were listed and allocated by one of us (L.M.) in one of the following categories: (i) a priori not at risk; (ii) a priori at risk; and (iii) a priori at high risk on the basis of Italian and international literature. The assessor has experience in exposure assessment and a deep knowledge of productive cycles. In Table I the a priori at risk jobs are listed. Accordingly, the following subjects were allocated in the exposed category: (i) those subjects who ever worked in a priori at risk job (including those who ever worked in an a priori at high-risk job); and (ii) those subjects who ever worked in an a priori at high-risk job. Concerning the second method, an industrial hygienist, specifically expert in exposure assessment, reviewed the complete occupational history and all the JSQs of each subject and assigned to each subject a level of intensity and probability of exposure. Intensity was defined in three categories (low, medium, high) and probability in four levels (zero, low, medium, high). Subjects who, following the evaluation of the industrial hygienist, experienced exposure to solvents with high or medium level of intensity and high or medium level of probability were considered as 'exposed to solvents' and they were compared with subjects who, following the judgement of the industrial hygienist, did not experience exposure with these levels of intensity and probability.

Statistical analysis

The design is a case–case study in which occupational exposures of patients with *ras*-positive AML were compared with those of *ras*-negative AML patients. The Student's *t*-test was used to assess differences in continuous variables. Crude and sex and age adjusted odds ratios were estimated for smoking habits, alcohol consumption, exposure to solvents and both X-ray and/or chemotherapy. A multivariate analysis taking into account age, sex, solvent exposure and X-ray and chemotherapy was also performed using the unconditional logistic regression. The Stata analysis software package was used (29). Concerning occupational exposures, we allocated in the exposed category: (i) subjects of who had ever worked in an a priori at risk job, including those subjects who worked in an a priori at high-risk job; or (ii) subjects of who had ever worked in an a priori at high-risk job; or (iii) subjects who were classified by the industrial hygienist as exposed to solvents with a medium or high level of intensity and probability of exposure based on the JSQ.

Table I. A priori at risk occupations in the 111 AML patients

ILO code[a]	Job
57020	Women's hair dresser[b]
79290	Fur tailor
80360	Leather goods assembler[b]
83220	Tool and die maker
83410	Machine-tool operator
84320	Automobile mechanic[b]
84340	Motor-cycle mechanic[b]
84970	Plant maintenance mechanic
87110	Pipe fitter
89590	Glass and ceramics painter and decorator[b]
90220	Tire builder[b]
92950	Textile printer[b]
93130	Structural steel and ship painter[b]
95415	Construction carpenter

[a]International Labour Office, 1968.
[b]A priori at high-risk occupations.

Table II. Frequency of N- and K-*ras* mutations in 111 AML patients[a]

Single mutations 14 (12.6%)			Double mutations 20 (18.0%)		
N-*ras* Codon 12 12 (10.8%)	K-*ras* Codon 12 1 (0.9%)	K-*ras* Codon 13 1 (0.9%)	N-*ras* Codons 12/13 16 (14.4%)	N- and K-*ras* Codon12 3 (2.7%)	N-*ras* Codon 13 K-*ras* Codon 12 1 (0.9%)

[a]No mutations were found in codon 61 of either N-*ras* or K-*ras*.

Results

Characteristics of the study population

During the 3-year study, a total of 111 AML patients were enrolled. Fifty-nine patients were enrolled in Turin, representing 81% of all incident cases in the city of Turin for the period of the study; 52 patients were enrolled in Florence and Prato areas, representing 70% of all incident cases in these areas. The diagnosis of AML was made according to the FAB classification. Mean age at diagnosis was 52.8 years old, and median age was 53 years old (SD 13.09). There were slight gender differences in the distribution of AML cases (52.3% males; 47.8% females). The most frequent FAB subtypes were M4 (34.9%) and M2 (23.9%), the mean peripheral blast count was 66.4% (median 79%; SD 29.5%) and the mean white blood cells count was 63.5×10^9/l (median 25.5×10^9/l; SD 114.4×10^9/l). Fifteen cases (13.5%) had a previous diagnosis of MDS. Seven patients out of 107 cases for whom the information was recorded (6.5%) had a previous history of chemotherapy and/or X-ray therapy.

ras mutations in AML patients

DNA samples were screened for the presence of N- and K-*ras* gene mutations. We found 34 AML patients positive for *ras* mutations (30.6%). None of the 15 healthy donors showed mutations in N- or K-*ras*. A total of 14 patients (12.6%) had a single *ras* mutation and 20 patients (18%) had two *ras* mutations (Table II). These mutations were in codons 12 and 13 of N- and K-*ras*, while no mutation was detected in codon 61. Among the 20 patients with two mutations, 16 patients had both mutations in N-*ras*, and four patients had one mutation in both N- and K-*ras* genes. All *ras* mutations were missense point mutations, and point mutations involving N-*ras* codons 12 and 13 were predominant, occurring in 28 of 34 mutation-positive patients. Transition G:C→A:T was the most frequent mutation, occurring in all 34 mutated cases, followed by transversion G:C→T:A, which was present in 10 of the mutation-positive cases (Table III).

Patients with *ras*-positive AML were older than *ras*-negative cases (mean age at diagnosis: 56.6 versus 51.3 years; P value = 0.05). Moreover, the frequency of *ras* activation in males was slightly higher than in females (34.5% for males, 26.4% for females). There were no differences in the FAB distribution between *ras*-positive and *ras*-negative patients (38.2% M4, *ras*-positive patients; 23.5% M2, *ras*-positive patients; 33.3% M4, *ras*-negative patients; 24% M2, *ras*-negative patients). The frequency of *ras* mutations was higher in patients with a previous MDS compared with those patients without a previous MDS [8/15 (53.3%) versus 26/96 (27.1%), P value = 0.04].

Occupational and other personal exposures and ras *mutations*

Results on the association between frequency of *ras* mutations and some personal and environmental exposures are shown in Table IV. No association was found between *ras* mutations and alcohol or smoking habits. Concerning occupational exposures, we found that *ras*-positive patients were more likely to have been employed in an a priori at risk occupation than *ras*-negative ones (OR = 3.6; 95% CI: 1.1–11.7). The association was stronger when high-risk occupations were considered, even if confidence intervals were wide (OR = 7.4; 95% CI: 0.6–390.9). When only the 20 patients with double *ras*-mutations were considered, the odds ratio was equal to 5.8 (95% CI: 1.5–21.2) for having ever worked in an a priori at risk job and 8.4 (0.4–504.4) for having ever worked in an a priori at high-risk job. When the industrial hygienist, based on information collected in the JSQ, assessed the exposure to solvents the odds ratio was equal to 1.5 (95% CI: 0.4–4.8).

Seven patients had undergone chemotherapy and/or X-ray for a previous malignancy (one NHL, four breast, one colon, one myelofibrosis); 100 patients did not receive any chemotherapy/X-ray; no information was available for four patients. The odds ratio for *ras* mutations and a previous X-ray and/or chemotherapy was equal to 16.2 (95% CI: 1.8–755.9, six exposed cases). For chemotherapy alone the odds ratio was equal to 5.4 (95% CI: 0.27–323, two exposed cases). When adjusted for age, sex and chemotherapy/X-ray in a multivariate analysis, the association between *ras* mutations and employment in an a priori at risk job did not change substantially. The odds ratio were equal to 2.8 (95% CI: 0.9–9.0) for having ever worked in a priori at risk job, 7.9 (95% CI: 0.7–94.4) for having ever worked in an a priori at high-risk job and 1.4 (95% CI: 0.4–4.6) for solvent exposure defined on the basis of the JSQ. When considering only subjects with double mutations the odds ratio were equal to 4.8 (95% CI: 1.2–18.8), 8.8 (95% CI: 0.4–179.1) and 1.2 (95% CI: 0.2–6.0) for having ever worked in an a priori at risk job, in an a priori at high risk and for solvent exposure as defined by the industrial hygienist, respectively. When only patients without previous chemotherapy were considered, the odds ratio for having worked in an a priori at risk job, adjusted by age and sex, was equal to 2.2 (95% CI = 0.6–7.3, based on six *ras*-positive exposed subjects) (data not shown).

Discussion

The members of the *ras* gene family encode for 21-kDa proteins (p21ras) that localize to the inner plasma membrane and have guanosine triphosphate (GTP)-binding and hydrolysis activity. Point mutations at codons 12/13 or codon 61 of *ras*

Table III. Characteristics of cases with N- or K-*ras* mutations

Age (years)/sex	FAB	Previous MDS	X-ray (R) Chemotherapy (C)	Codon affected	Base change	Amino acid substitution	Job[a]/exposure (JSQ)
51/M	M5			N12	GGT→GAT	Gly→Asp	Working proprietor (retail trade); Salesman
68/M	0 M0	Yes		N12 N13	GGT→GAT GGT→AGT	Gly→Asp Gly→Ser	Accountant; Teacher
58/F	M4			N12	GGT→GAT	Gly→Asp	Sewer
66/M	M5	Yes		N12 N13	GGT→GAT GGT→AGT	Gly→Asp Gly→Ser	**Fur tailor**; Store room clerk
70/F	M4			N12	GGT→GAT	Gly→Asp	Paper book maker
66/M	M4			N12 K12	GGT→GAT GGT→TGT	Gly→Asp Gly→Cys	Farmer; Railway and road vehicle loader
62/M	M4			N12	GGT→GAT	Gly→Asp	Weaver; Office clerk
66/F	M2		R	N12 K12	GGT→GAT GGT→TGT	Gly→Asp Gly→Cys	Housewife
52/F	M1			N12	GGT→GAT	Gly→Asp	Basketry maker; Knitting machine operator; **Shoe cutter and laster**; **Leather goods assembler**; **JSQ**+
37/M	M1			N12	GGT→GAT	Gly→Asp	Telephone operator
37/M	M4			N12 K12	GGT→GAT GGT→TGT	Gly→Asp Gly→Cys	**Machine-tool operator**; Construction painter; **JSQ**+
66/M	M2	Yes		N12	GGT→GAT	Gly→Asp	Boatmen
67/F	M2	Yes		N12	GGT→GAT	Gly→Asp	Housewife
66/M	M4			N12	GGT→GAT	Gly→Asp	**Machine tool operator**; Plastic ingestion machine operator; **JSQ**+
62/M	M2			N12	GGT→GAT	Gly→Asp	Farmer, Spinner
48/M	M2			K13	GGC→GAC	Gly→Asp	Cinema projectionist; Lawyer; Teacher
78/M	M4	Yes		K12 N13	GGT→AGT GGT→AGT	Gly→Ser Gly→Ser	Radio and television repairmen; Administration manager
61/M	M4			K12	GGT→AGT	Gly →Ser	Retail Trade salesman; Labourer; **JSQ**+
72/M	M0	Yes		N12 N13	GGT→GTT GGT→GAT	Gly→Val Gly→Asp	Commercial traveller; Farmer
46/F	M2			N12 N13	GGT→GCT GGT→GAT	Gly→Ala Gly→Asp	Sewer; **Fur tailor**; Nurse; Working proprietor (restaurant)
53/M	M4			N12 N13	GGT→GAT GGT→GAT	Gly→Asp Gly→Asp	Office clerk; Commercial traveller; Motor-vehicle driver
67/F	M4			N12 N13	GGT→GAT GGT→GAT	Gly→Asp Gly→Asp	Sewer; Labourer
71/F	M0	Yes		N12 N13	GGT→GAT GGT→GAT	Gly→Asp Gly→Asp	Garment cutter; Office clerk
42/M	M3			N12 N13	GGT→TGT GGT→GAT	Gly→Cys Gly→Asp	**Textile printer**; **JSQ**+
52/M	M3		C	N12 N13	GGT→GAT GGT→AGT	Gly→Asp Gly→Ser	**Tire builder**; Macaroni maker; Commercial traveller
46/M	M2		C	N12 N13	GGT→TGT GGT→AGT	Gly→Cys Gly→Ser	**Tool and die maker**; General foreman; Production manager; Office clerk; Commercial traveller; **JSQ**+
49/F	M4			N12 N13	GGT→GTT GGT→GAT	Gly→Val Gly→Asp	Nurse

Table III. *Continued*

Age (years)/sex	FAB	Previous MDS	X-ray (R) Chemotherapy (C)	Codon affected	Base change	Amino acid substitution	Job[a]/exposure (JSQ)
52/F	M5		R C	N12 N13	GGT→GAT GGT→GAT	Gly→Asp Gly→Asp	Farmer
63/M	M0			N12 N13	GGT→GTT GGT→GAT	Gly→Val Gly→Asp	Wood carver; **Glass and ceramic painter**; **JSQ+**
30/F	M2			N12 N13	GGT→GTT GGT→GAT	Gly→Val Gly→Asp	Teacher; Service worker; Journalist; Jurist
63/M	M4	Yes		N12 N13	GGT→GAT GGT→GAT	Gly→Asp Gly→Asp	Mosaic cutter; Mechanic assembler; **Plant maintenance mechanic**
66/F	M1		R C	N12	GGT→GAT	Gly→Asp	Service worker; Religious worker; Missionary
44/F	M3		R C	N12 N13	GGT→GAT GGT→GAT	Gly→Asp Gly→Asp	Farm helper; Storeroom clerk; Hosiery worker
26/F	M4			N12	GGT→GAT	Gly→Asp	Electronic computer operator; Office clerk

[a]Job at a priori risk are in boldface.
FAB, French–American–British classification; JSQ+ means that a level of intensity and probability of exposure to solvents more than low was assigned to the subject by the industrial hygienist on the basis of their JSQ.

Table IV. Odds ratios (OR) and 95% confidence intervals (95% CI) for some personal and environmental exposures (AML)

Exposure	*ras*-positive One or two ras mutations	*ras*-positive Two ras mutations	*ras*-negative No ras mutations	OR (95% CI) Ever ras versus no ras	OR (95% CI) Two ras versus no ras
	(Ever ras) *n* (%)	(Two *ras*) *n* (%)	(No *ras*) *n* (%)		
Smoking (ever ras versus no ras: 109 cases; two ras versus no ras: 95 cases)[a]					
Never	15 (44.1)	8 (40.0)	34 (45.3)	1	1
Ever	19 (55.9)	12 (60.0)	41 (54.7)	1.1 (0.4–2.6)	1.2 (0.4–3.9)
Alcohol (ever ras versus no ras: 111 cases; two ras versus no ras: 97 cases)					
Never	13 (38.2)	7 (35.0)	30 (39.0)	1	1
Ever	21 (61.8)	13 (65.0)	47 (61.0)	1.0 (0.4–2.6)	1.2 (0.4–3.9)
Chemotherapy and/or X-ray (ever ras versus no ras: 107 cases; two ras versus no ras: 93 cases)[b]					
No	27 (81.8)	14 (73.7)	73 (98.7)	1	1
Yes	6 (18.2)	5 (26.3)	1 (1.4)	16.2 (1.8–755.9)	26.1 (2.5–1248.9)
Chemotherapy only (ever ras versus no ras: 103 cases; two ras versus no ras: 90 cases)[c]					
No	27 (93.1)	14 (87.5)	73 (98.6)	1	1
Yes	2 (6.9)	2 (12.5)	1 (1.4)	5.4 (0.3–323.2)	10.4 (0.5–623.2)
To have worked in a 'a priori at risk' job (ever ras versus no ras: 111 cases; two ras versus no ras: 97 cases)					
Never	25 (73.5)	12 (60.0)	69 (89.6)	1	1
Ever	9 (26.5)	8 (40.0)	8 (10.4)	3.6 (1.1–11.7)	5.8 (1.5–21.2)
To have worked in a 'a priori at high risk' job (ever ras versus no ras: 111 cases; two ras versus no ras: 97 cases)					
Never	31 (91.2)	18 (90.0)	76 (98.7)	1	1
Ever	3 (8.8)	2 (10.0)	1 (1.3)	7.4 (0.6–390.9)	8.4 (0.4–504.4)
JSQ+ (ever ras versus no ras: 109 cases; two ras versus no ras: 95 cases)					
Never	27 (79.4)	16 (80.0)	64 (85.3)	1	1
Ever	7 (20.6)	4 (20.0)	11 (14.7)	1.5 (0.4–4.8)	1.5 (0.3–5.8)
To have worked in a 'a priori at risk' job (ever ras versus no ras: 111 cases; two ras versus no ras: 97 cases)[d]					
Never	25 (73.5)	12 (60.0)	69 (89.6)	1	1
Ever	9 (26.5)	8 (40.0)	8 (10.4)	2.8 (0.9–9.0)	4.8 (1.2–18.8)
To have worked in a 'a priori at high risk' job (ever ras versus no ras: 111 cases; two ras versus no ras: 97 cases)[d]					
Never	31 (91.2)	18 (90.0)	76 (98.7)	1	1
Ever	3 (8.8)	2 (10.0)	1 (1.3)	7.9 (0.7–94.4)	8.8 (0.4–179.1)
JSQ+ (ever ras versus no ras: 109 cases; two ras versus no ras: 95 cases)[d]					
Never	27 (79.4)	16 (80.0)	64 (85.3)	1	1
Ever	7 (20.6)	4 (20.0)	11 (14.7)	1.4 (0.4–4.6)	1.2 (0.2–6.0)

[a]Two subjects were excluded because without information on smoking habits.
[b]Four subjects were excluded because of no information on previous X-ray or chemotherapy.
[c]Three subjects with X-ray and chemotherapy and one patient with X-ray therapy alone were excluded.
[d]Adjusted for age, sex and X-ray and/or chemotherapy.
JSQ+ is assigned to those individuals who were considered exposed to solvents with a probability and intensity level greater than 'low' by the industrial hygienist.

gene family determine the replacement of specific amino acid residues in either the guanosine GTP-binding domain or in the GTPase domain of p21ras, respectively. Therefore, *ras* mutations lock p21ras in the active GTP-bound form resulting in activation of the constitutive protein and tumour development. Activation of *ras* genes shows considerable variability among different neoplasms (30), and N- and K-*ras* mutations occur in nearly 15–30% of AML cases (5–7). Our investigation of 111 patients with AML for the presence of N- and K-*ras* mutations led to the detection of 30.6% positive cases (34/111). This relatively high prevalence of *ras*-mutations may reflect the fact that we did not exclude cases with previous MDS, a related AML disorder in which *ras* gene mutations have been observed (14–17), and AML cases that have received prior X-ray or chemotherapy. Indeed cases with previous MDS and cases with prior exposure to chemotherapy and X-ray therapy that are classified as therapy-related AML (31,32), might contribute a larger share of *ras* mutations. The presence of two mutations in AML patients have already been reported by other investigators and have been explained by assuming that *ras* oncogene mutations might contribute to the outgrowth of different and more malignant sub clones of cells (13).

We found that *ras*-positive AML patients were more likely to have been employed in an occupation a priori at risk for solvent exposure than *ras*-negative ones (OR = 2.8; 95% CI: 0.9–9.0). The association was stronger when at high-risk occupations were considered, even though confidence intervals were extremely wide (OR = 7.9; 95% CI: 0.7–94.4); nevertheless, when considering exposure to solvents assessed by the industrial hygienist the risk fell to 1.4 (95% CI: 0.4–4.6). When considering as cases only the 20 patients with double mutations, the association with having worked in an 'a priori at risk job' was stronger and statistically significant (OR = 4.8; 95% CI: 1.2–18.8). One previous study by Taylor *et al*. (18) showed that *ras*-positive AML patients, rather than *ras*-negative AML patients, were more likely to have worked in jobs entailing exposure to chemical and physical hazards. This study found that, when *ras*-positive AML patients were compared with healthy controls, the odds ratio for all exposures to chemicals was >1, whereas patients with *ras*-negative AML showed no increased risk when compared with healthy control subjects. This study is the only one that used mutations in the *ras* proto-oncogene to classify subtypes of AML and showed that *ras*-positive AML patients were associated with exposure to chemicals; the results of Taylor's study raise the possibility that exposure to chemicals in the occupational environment induce *ras* mutations and that activation of the *ras* proto-oncogene is an initiating event in the leukaemogenesis process. Our results are consistent with this hypothesis, but the association between *ras* mutations and occupational exposure was not statistically significant and chance cannot be excluded. Another weakness of our study must also be pointed out, that is, different associations were obtained with the two different methods of classifying the exposure of the subjects. The association was stronger, even though not significant, when subjects who had worked in an a priori risk job were considered exposed to solvents than when the exposure was assessed by the industrial hygienist. One possible explanation might be that not solvents alone, but rather a more complex pattern of exposures that are brought about while doing a particular job, may be responsible for the *ras* activation effect. The selected occupations may involve exposures to other chemical substances, such as some dyes, polycyclic aromatic hydrocarbons, organic powder/dust, etc. Different results were also described in a study of exocrine pancreatic cancer patients in which an association between K-*ras* mutations and solvent exposure was observed when occupational exposure was defined based on the job/exposure matrix, but not when the exposure was assessed on individual basis by a panel of experts (33).

A strong statistically significant association between *ras* mutations and a previous X-ray and/or chemotherapy for a previous malignancy was found in our study. Our findings add evidence to the hypothesis that *ras* mutations might be the consequence of hazardous exposures. This result seems to be of great interest, given that patients who have received cytotoxic and X-ray therapy for primary neoplastic diseases have an increased risk of developing a therapy-related AML (31,32). In Taylor's study (20), out of 50 patients in complete remission from childhood acute lymphoblastic leukaemia, three patients (6%) were found to harbour N-12 *ras* mutations (one of these harboured a mutation at codon 969 of the FMS proto-oncogene, which encodes the cell surface receptor for colony stimulating factor 1) after therapy, whereas no *ras* mutations were detected in samples taken at the time of primary disease.

In our study transition G:C→A:T was present in all mutation-positive cases. This selective mutation has also been noted by other investigators (5,21). In addition, 10 of the 34 *ras*-positive cases had G:C→T:A transversions. G:C→A:T transitions and G:C→T:A transversions are considered to be suggestive of exposure to carcinogens (35,36). Moreover, G→A transitions at the second G of a GG pair are characteristic for exposures to alkylating agents (37). Indeed, in our study appeared a statistically significant association between previous exposure to chemotherapies, which employ potential methylating agents. Moreover, exposure to methylating agents can occur through diet or lifestyle, by the bacterial or chemical nitrosation of amines, or through occupational exposure. There is evidence that methylating agents induce the formation of damaged bases O^6-methylguanine. In the absence of DNA repair, O^6-methylguanine has been shown to induce G:C→A:T transition mutations (38,39). Therefore, it could be interesting to investigate whether inter-individual variation in DNA repair systems could be a susceptibility factor for development of AML after exposure to chemical agents. A recent study by Casorelli *et al*. suggested that defective recombinational DNA repair might promote the development of secondary leukaemia (40). In conclusion, our study is compatible with the hypothesis that the *ras* oncogene might identify subtypes of leukaemia that occur in people who had X-ray and/or chemotherapy for a previous malignancy or were occupationally exposed to chemical agents. However, the sample size of the study is small and the low frequency of occupational and of therapeutic exposures make odds ratios unstable and then hard to interpret. Further studies, also taking into account impairment of DNA repair system, seem to be necessary in order to confirm this hypothesis.

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