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Oncogene Amplifications, Rearrangements, and Restriction Fragment Length Polymorphisms in Human Leukaemia

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Abstract—*We have determined the prevalence of amplification and rearrangements for* c-myc, c-myb, c-mos, bcr, c-abl, c-Ha-ras-1, c-N-ras, *and* c-K-ras-2 *in a total of* 51 *cases of human leukaemia* (19 *patients with AML*, 13 *cases with CML*, 14 *cases with ALL, and* 5 *cases with CLL*). *Amplifications at a level of more than* 2 two copies *per haploid genome are apparently very rare and were found only once for* c-myb *in a c-ALL patient. Oncogene rearrangements were not found except for* bcr, *which was rearranged in all cases of CML, and* 5 *cases of ALL studied. Restriction fragment lengths polymorphisms (RFLPs) were also analysed. A previously described rare* 5 kb *EcoRI allele at the* c-mos *locus was absent in our patients. Rare alleles at the* c-Ha-ras-1 *locus were found to be significantly more prevalent in our patients than in a control group. Transfection experiments revealed no dominant transforming oncogenes in the tumour DNA of* 3 *patients carrying such rare alleles.*

INTRODUCTION

CELLULAR oncogenes, some of which are the cellular homologues of retroviral onc-genes, play key roles in embryonal development, cell proliferation and differentiation; in tumours, these genes can be damaged by rearrangements (due to inversion, deletion, insertion, or translocation of DNA), amplification, and mutations (in controlling or coding regions) [1]. Since these lesions are possibly related to the aetiology of the tumours in which they are found, they could also serve as molecular markers of specific diseases or subgroups of clinically indistinguishable entities. Here, we report on our first attempts to determine the feasibility of using oncogene amplifications and rearrangements in the classification of leukaemia.

Gene amplifications have been found in a number of different tumour types; especially interesting is the amplification of *N-myc* [2], and *L-myc* [3] genes, which have been correlated to the clinical stage and the progression of neuro- and retinoblastomas, and lung cancer, respectively. The *c-myc* and *c-myb* oncogenes have been suggested to control cell proliferation in the haematopoietic lineage [1], and were found to be amplified in promyeolocytic [4] and myelogeneous [5] leukaemias, respectively.

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Among the oncogene rearrangements detected, one of the most interesting cases is that of the *bcr* gene on chromosome 22q11. In chronic myelogeneous leukaemia, the *bcr* locus is rearranged due to the invasion of the *c-abl* gene normally residing on chromosome 9q34 [6].

Recent data indicate that some oncogene loci display restriction fragment length polymorphisms (RFLP) and that some alleles are associated with tumour susceptibility [7, 8].

To exploit such genetic alterations for clinical purposes, we systematically investigated the presence of gene amplifications and rearrangements for *c-myc*, *c-myb*, *c-mos*, *bcr*, *c-abl*, *c-Ha-ras*-1, *c-N-ras*, and *c-K-ras*-2 in a total of 51 cases of untreated human leukaemia. In addition we also investigated the presence of RFLPs at the *c-mos* and the *c-Ha-ras*-1 loci. Our aim was to determine whether such genetic lesions could be used in the genetic classification of these tumours or not.

MATERIALS AND METHODS

Patients

All patients were studied at the time of diagnosis or before starting anti-leukaemic therapy. This precaution was taken since anti-neoplastic drugs are known to induce gene amplifications [9].

Table 1. *Characterisation of DNA probes used in this study*

Target sequence	DNA probe	Hybridising fragments (kb) EcoRI	BamHI	BglII	Ref.
*c-Ha-ras-1**	pUCEJ6.6	30	6.9	7.5	15
c-Ki-ras-2	p640	2.4	ND†	ND	20
c-N-ras	p6aI	ND	8.5	14.0	21
c-myb	pHM2-B	2.4	6.6	6.0	22
c-myc	pE3	13.0	ND	5.5	23
c-mos	pHM2A	2.5	ND	ND	24
bcr	3'-*bcr*	ND	3.4	5.0	6
c-abl	pAB1sub9	ND	ND	1.8/4.0/4.6	25
β-globin	pSS737	ND	1.8		26

*Note the presence of RFLP at this locus.
†ND: not performed in the present study.

DNA biochemistry and hybridisation analysis

DNA was extracted from leukaemic cells by established methods [10]. After complete digestion with restriction endonucleases (Boehringer Mannheim), DNAs were run through agarose gels in TBE, and blotted onto nylon membranes (Hybond N, Amersham) according to the method of Southern [11]. DNAs were fixed by u.v. cross-linking and hybridised according to established procedures [10] against gel-purified DNA-fragments labelled to high specific activities (1×10^9 cpm/μg) with (^{32}P)dCTP (Amersham) using the method of Feinberg and Vogelstein [12]. After washing to a final stringency of 0.2 × SSC at 68°C, the filters were exposed to Kodak X-Omat X-ray film with intensifying screens for 16 hr.

Immunogenotyping analysis was performed as described [13]]. The DNA probes used in the present study are detailed in Table 1.

Transfection assays

Transfection assays were performed with 3×10^6 NIH3T3 cells and 40 μg of tumour DNAs by electroporation as described by Potter *et al.* [14] using an electroporation device made in our workshop (construction details are available upon request from the authors). A negative control sample consisted of human placental DNA, a positive control was made by the addition to placental DNA of one genome equivalent of the cloned EJ *c-Ha-ras-1* gene, which carries a point mutation in codon 12 of the p21 ras protein [15]. Morphologically-altered foci were evaluated after 4 weeks, and replated into soft agar.

RESULTS

1. *Oncogene amplifications and rearrangements*

Our strategy to investigate the presence of amplifications and rearrangements was based on Southern blot hybridisation experiments using oncogene-specific probes (Table 1) after digestion of DNA extracted from leukaemic cells with various restriction enzymes. In the search for gene amplification an internal control for the amount of DNA available for hybridisation on the filters was provided by co-hybridisation to β2-microglobulin or β-globin gene probes. The degree of hybridisation was thus standardised and evaluated by densitometric scanning. As a threshold for amplification, we set a level of 4 copies of a gene per genome. Using this criterion, *c-myc*, *c-mos*, *bcr*, *c-abl*, *c-Ha-ras-1*, *c-N-ras*, and *c-Ki-ras-2*, respectively, were not amplified in the leukaemias studied here. A single case of amplification for the *c-myb* oncogene was found in the blast cells of a cALL patient (Fig. 1). The same experiment also demonstrated the absence of oncogene rearrangements with the exception of *bcr*. Rearrangements at *bcr* are the molecular characteristic of Ph+ CML [6], substantiated here for all of the 13 CML patients studied (Fig. 2a); interestingly, *bcr* rearrangements were found also in 5 out of 14 cases of ALL studied (Fig. 2b). Immunogenotyping analysis revealed the presence of cALL in these cases; in 2 of these cases, karyotype analyses were possible and revealed the presence of a Philadelphia chromosome (Boehm *et al.*, manuscript submitted for publication).

In conclusion, the present data indicate that oncogene amplifications and rearrangements are exceedingly rare in fresh leukaemia samples and are thus probably not involved in the genesis of disease.

Rearrangements at *bcr* on chromosome 22q11, however, are present in all cases of Ph+ CML, and in a substantial proportion of ALL patients.

2. *Oncogene RFLPs*

We sought to determine whether previously described rare alleles at the *c-mos* and the *c-Ha-ras-1* locus would also be present in our leukemia sample. Figure 4 indicates that the variant EcoRI allele of

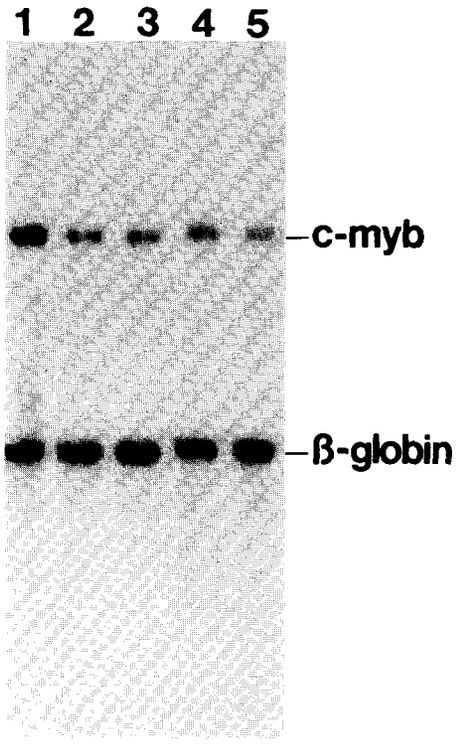

Fig. 1. *Amplification of* c-myb *in acute lymphoblastic leukaemia. BamHI digests were simultaneously hybridised with a* c-myb *and a* β-*globin gene probe. Patients analysed were as follows: lane* 1, *c-ALL; lane* 2, *AML; lane* 3, *T-ALL; lane* 4, *c-ALL; lane* 5, *T-ALL. By densitometric scanning of autoradiographs, the degree of amplification of* c-myb *in lane* 1 *was estimated to be about* 6–8.

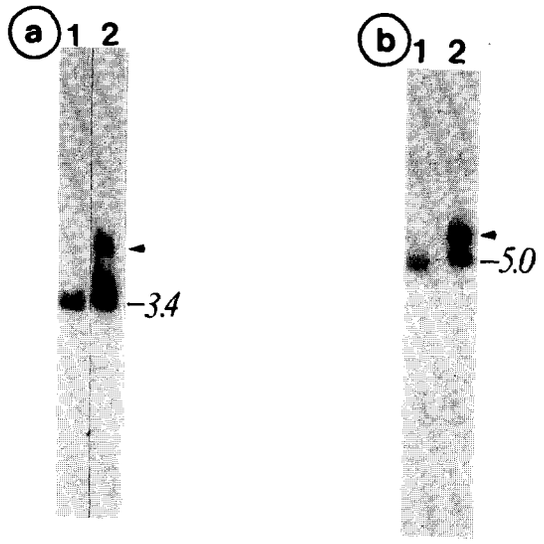

Fig. 2. *Rearrangement of* bcr *in CML and ALL. Panel* (*a*) *shows BamHI digested DNA isolated from a patient in chronic phase of CML* (*lane* 2) *together with a normal control* (*lane* 1). *Panel* (*b*) *displays the Bgl II digest of DNA from a patient with c-ALL* (*lane* 2) *together with a normal control* (*lane* 1). *Unrearranged alleles are indicated by a bar, rearranged fragments by an arrowhead. Numbers to the right of the lanes are sizes in* kb.

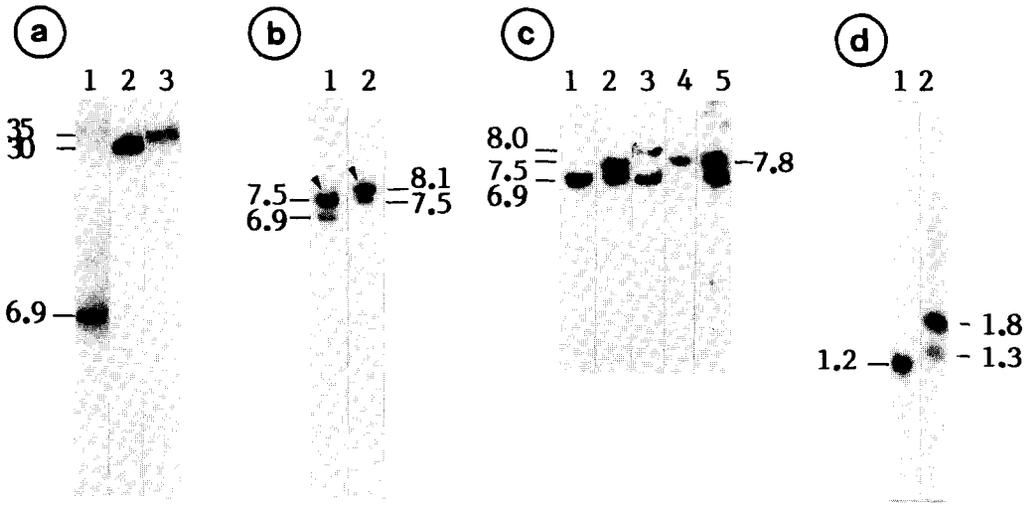

Fig. 3. *Presence of rare alleles at the* c-Ha-ras-1-1 *locus in leukaemia patients.* (*a*) *Hybridisation analysis of a normal individual after digestion of DNA with BamHI* (*lane* 1). *EcoRI* (*lane* 2), *and HindIII* (*lane* 3). (*b*) *Hybridisation analysis of DNA from a heterozygous individual after BamHI* (*lane* 1), *and Bgl II* (*lane* 2) *digestions. Note the stronger hybridisation in the upper fragment* (*arrowhead*). (*c*) *Hybridisation analysis of* 5 *leukaemia patients after digestion of DNA with BamHI.* (*d*) *Hybridisation analysis of DNAs from* 2 *leukaemia patients after HpaII plus MspI digestion. The numbers to the left and right of the lanes indicate fragment size in* kb.

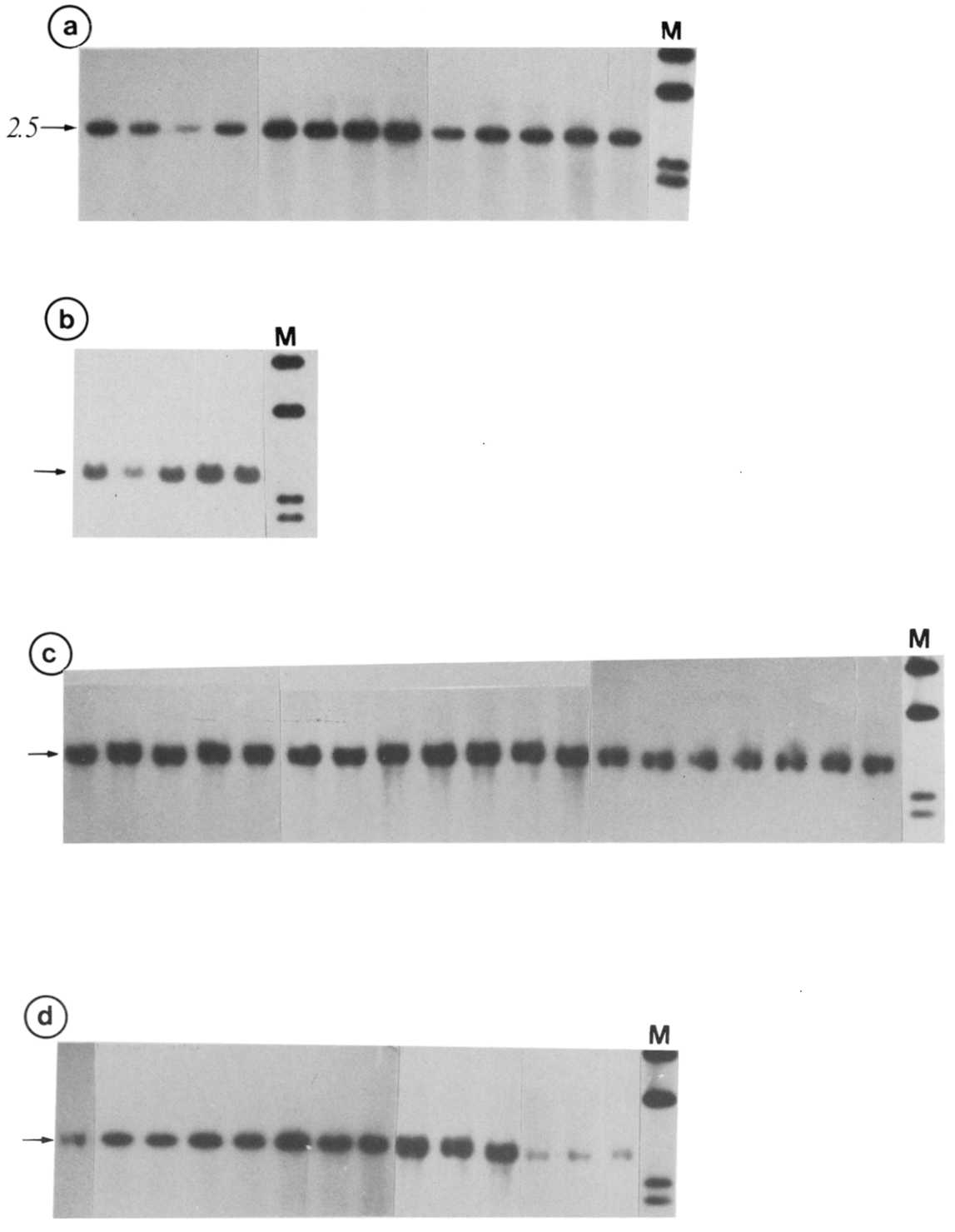

Fig. 4. Lack of a 5.0kb *EcoRI allele in the human* c-mos *locus in leukaemia patients. Displayed are the results obtained with CML (a), CLL (b), AML (c), and ALL (d) patients. Lanes to the right contain molecular weight markers. To the left, the size of EcoRI fragments is given in* kb.

Table 2. Frequency of alleles at the human c-Ha-ras-1 *locus*

Allele* (kb)	Control population†	Leukaemia population‡
Frequent alleles		
6.9	47/70 (0.67)	58/90 (0.64)
7.5	15/70 (0.22)	14/90 (0.15)
8.0	8/70 (0.11)	6/90 (0.07)
Rare alleles§		
7.0	0	3/90 (0.03)
7.1	0	1/90 (0.01)
7.8	0	8/90 (0.09)

*After BamHI digestion; all results were confirmed by HpaII plus MspI digestions, respectively.

†The control population consisted of healthy blood donors; the analysis of genotypes in the 2 populations revealed no significant deviation from the expected Hardy–Weinberg distribution.

‡The leukaemia population consisted of 20 AML, 14 ALL, 8 CML, and 3 CLL patients; a total of 6 patients displayed 1 rare allele in addition to 1 of the frequent alleles; 3 patients had 2 rare alleles at the locus.

§The presence of rare alleles in the leukaemia vs. the control populations is significantly different at $P < 0.01$ by Chi-square test.

5.0 kb at the human *c-mos* locus [7] was not observed in the 51 patients studied here. Thus, this allele is exceedingly rare in the leukaemia population and may thus not significantly contribute to disease susceptibility.

Krontiris *et al.* [8] have shown that the *c-Har-ras-1* locus is a multi-allelic system and that alleles rarely found in a normal population are significantly more prevalent in tumour patients. This finding was disputed in a recent report [16].

We therefore determined the proportion of rare *c-Ha-ras-1* alleles in our leukaemia population. Figure 4 exemplifies the presence of rare alleles at the *c-Ha-ras-1* locus.The individual analysed in Fig. 4(a) is homozygous for the most prevalent *c-Ha-ras-1* BamHI allele of 6.9 kb. The hybridisation after digestion with EcoRI and HindIII, respectively, was used in order to ascertain that the *c-Ha-ras1* locus was not rearranged in individuals with other BamHI alleles. For a more accurate measurement of the variable tandem repeat (VTR) present in this locus, double-digestion with HpaII and MspI can be applied (Fig. 4d). We consistently observed a higher hybridisation intensity with alleles other than the 6.9 kb BamHI fragment, i.e. those with a longer VTR sequence (Fig. 4b). This behaviour is due to the repetitive nature of this sequence, which is contained in our probe. The nature and the frequencies of the 2 classes of alleles at the *c-Ha-ras-1* locus are shown in Table 2. These data are based on both BamHI and HpaII plus MspI digestions, respectively, since it is not always possible to determine the presence of minor variants by BamHI digestion alone. To simplify the presentation, only the results for BamHI analyses are given. In the 35 healthy individuals studied, only 3 alleles were observed. In the leukaemia sample studied here, additional alleles were observed in 9 out of 45 patients (Table 2, Fig. 4c). Table 2 indicates that such rare alleles might indeed be more prevalent in leukaemia patients as compared to a normal control population of healthy blood donors. There appeared to be no association between any allele and a specific disease type. These data support the conclusion that rare alleles at this locus may predispose their carriers to leukaemia [8].

To investigate the possible nature of this predisposition, we tested for the presence of dominant transforming oncogenes in the leukaemic DNA of these patients by use of the transfection assay described in Materials and Methods. DNAs from 3 cases carrying rare alleles were found to be negative in this assay, whereas 3 out of 7 DNAs containing prevalent alleles elicited transformation of NIH 3T3 cells. Thus, there appears to be no obvious correlation between the presence of rare alleles and dominant transforming genes detectable by the NIH3T3 assay.

DISCUSSION

In this study, we set out to determine the prevalence of oncogene amplification and rearrangements in human leukaemia in order to see whether such alterations are sufficiently frequent to warrant their consideration for diagnostic purposes. In particular, we wished to determine whether some of the oncogenes implicated in control of proliferation are involved in such events. Precedence for oncogene amplification is the *N-myc* gene in neuroblastomas [2] and the *L-myc* gene in small-cell lung cancer [3]. The *c-myc* gene is believed to be involved in proliferation control in the haematopoietic lineage [1] and deregulation of its activity could also involve gene amplification. This is, however, not the case for most leukaemias studied at the time of diagnosis as presented here. Amplification of *c-myc* has mostly been found in established cell lines [1] and it is conceivable that propagation of leukaemia cells *in vivo* directly or indirectly facilitates gene amplification processes. This is also suggested by the finding that although *c-myc* gene amplification was observed in 2 of 11 transplantable stomach cancers in nude mice, no such amplification was detected in 19 samples of primary stomach cancer [17].

The fact that we have observed *c-myb* amplification in a patient with cALL is in contrast to an earlier report of *c-myb* amplification in an AML patient [5]. This points to a lower degree of tissue-specificity of *c-myc* and *c-myb* oncogenes or to their ability to complement each other as proliferation genes in the haematopoietic lineage. We were unable to correlate this gene amplification with *c-*

myb expression, since no RNA was available from these leukaemic blasts. Thus, it appears that oncogene amplification is exceedingly rare in fresh human leukaemias and can thus not serve as a basis for genetic classification of this disease. In addition, it is improbable that gene amplification is related to the genesis of these tumours. This mechanism, however, could well contribute to disease progression. This is exemplified in neuro- and retinoblastomas and lung cancers, where amplifications of the *N-myc* [2] and *L-myc* [3] genes, respectively, have been found to correlate to clinical stages of disease. Therefore, we are presently evaluating the frequency of *c-myc* and *c-myb* amplifications during progression of disease in order to see, for example, whether there is any correlation between evolving gene amplification events and response to therapy.

The lack of oncogene rearrangement in our leukaemia sample also precludes its use in genetic classification of these tumours. With the exception of *bcr* rearrangements, which appear to be specific for CML [6], but can also be observed in ALL patients, no other oncogene apparently suffers major structural changes at least at the time of diagnosis of leukaemia. Our detection of *bcr* rearrangements also in ALL indicates that this molecular marker is not entirely specific for CML. The use of *bcr* rearrangements in classification of acute leukaemias will be described in more detail elsewhere (Boehm *et al.*, manuscript submitted for publication).

The recent demonstration that some oncogene RFLPs may be associated with cancer susceptibility [7, 8] is of considerable theoretical and practical importance. A rare allele at the *c-mos* locus was found in patients with breast cancer and leukaemias [7] but was absent in our sample of leukaemia patients. In contrast, we were able to confirm the over-representation of rare alleles at the *c-Ha-ras*-1 locus in our leukaemia patients, which has been the subject of contradictory reports [8, 16]. It remains unclear at present, what the nature of tumour susceptibility associated with these variant *c-Ha-ras*-1 oncogene alleles is. It could be possible that the structural alteration itself is related to this phenomenon. However, the activity of the *c-Ha-ras*-1 for transformation of rodent cells is apparently not universally affected by the presence or absence of the 3′ region of the gene containing the VTR sequence [18, 19]. Here, we have tested whether carriers of rare alleles at the *c-Ha-ras*-1 locus are predisposed in leukaemia cells to activating mutations in dominant transforming oncogenes. Our data based on the NIH3T3 transfection assay indicate that there is no such correlation. Another possibility to explain the apparent tumour susceptibility of individuals carrying rare alleles at oncogene loci is that they may be in linkage disequilibrium with other genes (i.e. would then represent mere haplotype markers), which are themselves directly or indirectly related to disease susceptibility.

In conclusion, the data is this paper indicate that amplification and rearrangements of *c-myc*, *c-myb*, *c-mos*, *c-abl*, *c-Ha-ras*-1, *c-N-ras*, and *c-K-ras*-2 are extremely rare in fresh human leukaemias and are thus not useful in their genetic classification at the time of diagnosis. An exception is the *bcr* locus which is characteristically rearranged in CML, and is also altered in certain ALL patients, usually of the cALL phenotype. A RFLP at the *c-Ha-ras*-1 locus may be related to disease susceptibility, but the underlying mechanism remains obscure.

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