Point Mutation of the ras Protooncogenes and Chromosome Aberrations in Acute Nonlymphocytic Leukemia and Preleukemia Related to Therapy with Alkylating Agents

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ABSTRACT

Nine cases of overt acute nonlymphocytic leukemia and four cases of preleukemia or a myelodysplastic syndrome, all related to intensive treatment with alkylating agents, were studied cyogenetically and investigated using a rapid and sensitive dot blot screening procedure for point mutations in the Ha-ras, Ki-ras, and N-ras protooncogenes within codons 12, 13, and 61. The technique involves a selective amplification of genomic DNA sequences containing the codon sequence of interest, in combination with oligonucleotide hybridization. Examining fractionated mononuclear cells from bone marrow or peripheral blood, an N-ras mutation at position 13 was observed in one patient with overt leukemia, resulting in a base change from GGT to TGT thus converting glycine to cysteine. The other cases exhibited no ras gene mutations. It is surprising that c-ras mutations are only occasionally observed in overt acute nonlymphocytic leukemia related to treatment with alkylating agents, as such abnormalities have often been observed in acute nonlymphocytic leukemia de novo, and as many alkylating agents are known to produce DNA adducts leading to point mutations and substitution of single amino acids. The fact that deletions of varying parts of the long arms of chromosomes 5 and 7 are observed in most cases of therapy-related acute nonlymphocytic leukemia and preleukemia, as confirmed by our own series of 71 patients, suggests that loss of heterozygosity for specific alleles on the two chromosomes, rather than activation of a protooncogene, could be an important step in leukemogenesis.

INTRODUCTION

During the last few years two general types of genetic abnormality have been supposed to play a key role in the pathogenesis of human cancer, as described in recent reviews (1, 2). These abnormalities are activation of normal cellular genes (so-called protooncogenes) to dominant transforming genes by alteration of expression or structure, and the loss or inactivation of recessive putative repressor genes or antioncogenes.

A point mutation in the ras family of protooncogenes was first demonstrated in human bladder carcinoma cell lines (3, 4). A base change was demonstrated within codon 12 of the c-Ha-ras, resulting in the conversion of glycine to valine in the encoded p21 protein. This small alteration resulted in transforming properties of the protein when examined by the NIH 3T3 cell assay. Since then several studies have shown the presence of point mutations in the Ha-ras, Ki-ras, and N-ras protooncogenes of various human malignancies, in solid tumors (5–8) as well as in acute nonlymphocytic leukemia (9–13) or myelodysplasia (14). From those latter studies it can be suggested that the N-ras protooncogene is activated in more than 50% of patients suffering from acute nonlymphocytic leukemia. Alkylating agents are known to cause chromosome aberrations and DNA damage. Some alkylating agents form DNA adducts, such as O6-alkylguanine, which may lead to miscoding at replication (15). Mammary carcinomas have been induced in animal experiments by nitrosomethylurea, and a specific point mutation in the Ha-ras protooncogene in the tumor tissue has been implicated directly in carcinogenesis (16). It would therefore be of particular interest to study a human tumor type induced by alkylating agents, for possible activation of the ras protooncogenes by point mutation.

Therapy-related or secondary acute nonlymphocytic leukemia is such a type of tumor, which in several studies has now been related intimately to therapy with alkylating agents (17–25). The relative risk (ratio of observed/expected cases) of acute nonlymphocytic leukemia has varied in these studies between 67 and 175, indicating that statistically only one case in 67–175 cases of therapy-related leukemia can be expected to arise de novo. All the other cases must be considered as therapy induced, demonstrating the high specificity of this particular disorder.

We here report results of a study examining point mutations within codons 12, 13, and 61 of the Ha-ras, Ki-ras, and N-ras protooncogenes in nine patients with overt acute nonlymphocytic leukemia following treatment with alkylating agents, and in four patients with therapy-related preleukemia. In addition, the most important clinical and cytogenetic data of the patients are provided. These results increase our total series of patients with therapy-related leukemia or preleukemia studied cyogenetically from 78 to 88 cases, and the number of banded cases from 61 to 71.

MATERIALS AND METHODS

Patients. Cells were studied from bone marrow or peripheral blood of nine patients with overt acute nonlymphocytic leukemia and four patients with preleukemia, all following previous treatment of other malignant diseases with alkylating agents. In only two of these patients, cases 58 and 71, have the clinical and cytogenetic results been published previously (26). The most important clinical and cytogenetic characteristics of the patients are shown in Tables 1 and 2. Four patients had been treated for Hodgkin’s disease, three for non-Hodgkin’s lymphomas and five for different solid tumors. One patient (case 58) was examined in the preleukemic phase as well as after developing overt leukemia. The latent period from start of treatment with alkylating agents to development of preleukemia or leukemia varied between 14 and 104 months. The percentage of blasts in the cell source used for the experiments (bone marrow or peripheral blood) varied between 19 and 86% for patients with overt leukemia, and between 1 and 13% for patients with preleukemia.

Isolation of Cells. Mononuclear cells from blood or bone marrow were isolated over a Ficoll-Paque gradient. The cells were then washed three times with RPMI 1640 with 0.2% sodium hydrocarbonate, resuspended, frozen directly, and stored on liquid nitrogen at −195°C until the present investigation.

DNA Isolation and Examination. Genomic DNA was isolated by standard proteinase-K treatment and phenol-chloroform extraction...
Table 1 Clinical, cytological, and cytogenetic characteristics of patients with overt therapy-related acute nonlymphocytic leukemia examined for point mutations in the Ha-ras, Ki-ras, and N-ras protooncogenes at amino acid positions 12, 13, and 61

<table>
<thead>
<tr>
<th>Case</th>
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<th>Primary tumor histology/stage</th>
<th>Treatment for primary tumor</th>
<th>Status of primary tumor</th>
<th>Latent period to leukemia, (months)</th>
<th>Preleukemic phase duration, (months)</th>
<th>Survival (months)</th>
<th>Cytogenetic characteristics, bone marrow</th>
<th>Mitosis, abnormal/normal</th>
<th>Origin of cells % blasts</th>
<th>FAB-type*</th>
</tr>
</thead>
<tbody>
<tr>
<td>79</td>
<td>39/M</td>
<td>Hodgkin NS/IIIA</td>
<td>X-rays mantle CVPP (6 mo)</td>
<td>CR</td>
<td>48</td>
<td>11</td>
<td>5*</td>
<td>45,XY,−5,+11,−17,−19,20q+,+t(5p17q)</td>
<td>24/0</td>
<td>Marrow 30%</td>
<td>M1</td>
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<tr>
<td>80</td>
<td>57/M</td>
<td>Hodgkin NS/IIIA</td>
<td>CVPP (6 mo)</td>
<td>CR</td>
<td>45</td>
<td>3</td>
<td>3</td>
<td>45,XY,−6,+11,+t(11p13q);(p12 or 13;7)</td>
<td>24/7</td>
<td>Marrow 73%</td>
<td>M1</td>
</tr>
<tr>
<td>81</td>
<td>78/M</td>
<td>Non-Hodgkin Lymphocytic/IVA</td>
<td>Chl (4 mo), Ctx + VCR + Pred (29 mo)</td>
<td>CR</td>
<td>32</td>
<td>3</td>
<td>17+t(11q17q),i(13q)</td>
<td>34/0</td>
<td>Marrow 86%</td>
<td>Blood 45%</td>
<td>M1</td>
</tr>
<tr>
<td>58a</td>
<td>49/M</td>
<td>Non-Hodgkin DH/IIA</td>
<td>X-rays mantle VCR + Pred + Stn (2 mo) Ctx + VCR + Pred (22 mo)</td>
<td>CR</td>
<td>107</td>
<td>56</td>
<td>6</td>
<td>46,XY,−7,−mar</td>
<td>26/0</td>
<td>M4 blood 55%</td>
<td>M5b</td>
</tr>
<tr>
<td>82</td>
<td>58/F</td>
<td>Non-Hodgkin NH/I A</td>
<td>X-rays mantle Ctx + VCR + Pred (12 mo)</td>
<td>CR</td>
<td>18</td>
<td>12*</td>
<td>46XX</td>
<td>0/22</td>
<td>Marrow 60%</td>
<td>M4</td>
<td></td>
</tr>
<tr>
<td>83</td>
<td>50/F</td>
<td>Ovarian cancer Cystadeno-carcinoma III</td>
<td>Ctx + 5FU + Adm (8 mo), Cis Plat + HMM (10 mo)</td>
<td>PD</td>
<td>24</td>
<td>6</td>
<td>46,XX,t(9;11)(p21;q23)</td>
<td>23/0</td>
<td>Marrow 23%</td>
<td>M5b</td>
<td></td>
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<tr>
<td>84</td>
<td>59/F</td>
<td>Ovarian cancer Cystadeno-carcinoma I b</td>
<td>Ctx (6 mo), Ctx + 5FU + Adm (6 mo), Cis Plat + HMM (8 mo)</td>
<td>PR</td>
<td>92</td>
<td>2</td>
<td>2*</td>
<td>45,XX,−3,−7,−der(3)(3q24 or q25 or q26?)</td>
<td>14/8</td>
<td>Marrow 23%</td>
<td>M1</td>
</tr>
<tr>
<td>71</td>
<td>62/M</td>
<td>Apudoma (liver+lung)</td>
<td>CCNU + VCR + Ctx + VP16 (4 mo), Streptz (4 mo)</td>
<td>PD</td>
<td>14</td>
<td>3</td>
<td>11</td>
<td>46,XY,del(7)(q22)</td>
<td>27/0</td>
<td>Marrow 23%</td>
<td>M2</td>
</tr>
<tr>
<td>85</td>
<td>78/M</td>
<td>Rectal cancer disseminated</td>
<td>BCNU + 5FU + VCR + DTIC (24 mo)</td>
<td>CR</td>
<td>104</td>
<td>1</td>
<td>46, XY</td>
<td>0/22</td>
<td>Marrow 19%</td>
<td>M4</td>
<td></td>
</tr>
</tbody>
</table>

* Examined in overt leukemia.

**FAB, French-American-British; NS, nodular sclerosis; DH, diffuse histiocytic; NH, nodular histiocytic; CVPP, lumostine + vinblastine + procarbazine + prednisone; Chl, chlorambucil; Ctx, cyclophosphamide; VCR, vincristine; Pred, prednisone; Stn, streptonigrin; 5FU, 5-fluorouracil; Adm, doxorubicin; Cis Plat, cisplatinum; HMM, hexamethylmelamine; CCNU, lumostine; VP16, vepecid; Streptz, streptozotocin; BCNU, carmustine; DTIC, dacarbazin; CR, complete remission; PD, progressive disease; PR, partial remission + still in live.
techniques. Analyses were performed for mutations within codons 12, 13, and 61 in the three ras genes (Ha-, Ki-, and N-ras) by means of a rapid sensitive detection assay. The DNA regions of interest were amplified in vitro with the polymerase chain reaction procedure, spotted onto nylon membranes, and analyzed for base pair substitutions by means of oligonucleotide hybridization (27).

Synthetic Oligonucleotide Probes. The oligonucleotides (20-mers) were prepared by one of us (J. L.) by an asynchronous simultaneous synthesis strategy (28) using the solid-phase phosphite triester method. A complete list of oligomers used for the analyses of ras gene mutations is available on request. The oligomers were end-labeled using [γ-32P]-ATP (New England Nuclear, Boston, MA) and T4-polynucleotide kinase (Pharmacia). The kinased probes were separated from the unincorporated ATP by chromatography on Sephadex G 50 in 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Specific activities were greater than 2 x 10^6 cpm/pmol.

Polymerase Chain Reaction. DNA amplification in vitro was performed as described by Saiiki et al. (29) and Mullis and Faloona (30). The reaction mixture contained 150 ng chromosomal DNA, 100 ng of each of the amplimers (oligomers used for the chain elongation), 1 mM of each of the dNTPs, 10 mM Tris-HCl, pH 7.5, 10 mM MgCl2 in a total volume of 30 μl. The mixture was incubated for 5 min at 95°C to denature the DNA and the amplimers were allowed to anneal to the DNA at 37°C for 90 s. The polymerase chain elongation reaction was initiated by the addition of 0.5 μl (1 unit) of cloned Klenow polymerase (Pharmacia) and the mixture was incubated at 37°C for 90 s. A new cycle of amplification was started by denaturing the DNA for 90 s at 95°C. Routinely we perform 15 rounds of amplification with an outer set of amplimers followed by 15 rounds of amplification using an inner set of amplimers (30).

Dot Blot Hybridization. 1 μl of the DNA amplified in vitro was spotted onto nylon filters (Gene Screen Plus; New England Nuclear, Boston, MA). The filters were subsequently illuminated with a 254-nm UV lamp (1.6 kJ/m2) to bind the DNA to the filter. The filters were prehybridized overnight at 50°C in 5× SSPE, 7% SDS, 100 μg/ml sonicated, denatured salmon sperm DNA and 5× Denhardt’s solution and subsequently hybridized for 3 h at 50°C in the same mixture containing approximately 1 ng 32P-labeled oligomer probe. The filters were washed twice in 2× SSPE, 0.1% SDS for 5 min at room temperature, followed by a 30-min wash at 50°C in 5× SSPE, 0.1% SDS. Subsequently, the filters were washed stringently in 5× SSPE, 0.1% SDS for 10 min. Finally, the filters were exposed to Kodak XAR films at -70°C using intensifying screens.

Chromosome Studies. Cytogenetic investigations were carried out using GTL banding technique following a 1–4 day culture of a bone marrow sample. Results were expressed in accordance with the International System for Human Cytogenetic Nomenclature (ISCN, 1985) as previously described (26).

RESULTS

Among the nine patients with overt acute nonlymphocytic leukemia only one, case 80, presented a N-ras mutation within codon 13 with a base change from GGT to TGT. This mutation results in conversion of glycine to cysteine in the encoded protein (Fig. 1). None of the other eight cases of overt leukemia and none of the four cases with preleukemia exhibited any mutations at positions 12, 13, and 61 of the Ha-ras, Ki-ras, and N-ras protooncogenes (data not shown).

Cytogenetic studies of the bone marrow (Tables 1 and 2) revealed a -7 in two patients, 7q- in two patients (break points 7q22) and, interestingly, in case 87, two independent clones, one with -7 and one with 7q- (break point 7q11). One patient presented an unbalanced translocation t(5p17q), another a t(11q17q), the first leading to loss of the whole long arm of chromosome 5 and both translocations to loss of the short arm of chromosome 17. Finally, case 88 presented a 21q+ with break at 21q22, all findings highly characteristic for therapy-related leukemia. Five patients showed various abnormalities of chromosome 11, one of these was a t(9;11)(p21;q23). Two patients presented a completely normal karyotype. Interestingly, the patient with the N-ras mutation within codon 13 (case 80) presented normal chromosomes 5 and 7 and cytogenetic abnormalities atypical for therapy-related leukemia.

DISCUSSION

The present study demonstrates that specific point mutations in the ras protooncogenes may occur in overt acute nonlymphocytic leukemia only one, case 80, presented a N-ras mutation within codon 13 with a base change from GGT to TGT. This mutation results in conversion of glycine to cysteine in the encoded protein (Fig. 1). None of the other eight cases of overt leukemia and none of the four cases with preleukemia exhibited any mutations at positions 12, 13, and 61 of the Ha-ras, Ki-ras, and N-ras protooncogenes (data not shown).

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Table 2 Clinical, cytological, and cytogenetical characteristics of patients with therapy-related preleukemia examined for point mutations in the Ha-ras, Ki-ras, and N-ras protooncogenes at amino acid positions 12, 13, and 61.

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<th>Status of primary tumor</th>
<th>Latent period to leukemia (months)</th>
<th>Survival (months)</th>
<th>Cytogenetic characteristics, bone marrow</th>
<th>Mitoses abnormal/normal</th>
<th>Origin of blasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>86</td>
<td>48/M</td>
<td>Hodgkin's NS/IIA</td>
<td>X-rays mantle</td>
<td>CR</td>
<td>51</td>
<td>52</td>
<td>46,XYdel(11)(q14q23)</td>
<td>25/0</td>
<td>Marrow 1%</td>
</tr>
<tr>
<td>87</td>
<td>25/M</td>
<td>Hodgkin's MC/III A</td>
<td>MOPP (6 mo)</td>
<td>CR</td>
<td>18</td>
<td>22</td>
<td>45,XY,−7=13/46,XY,del(7)(q11)=5/0</td>
<td>18/8</td>
<td>Marrow 4%</td>
</tr>
<tr>
<td>88</td>
<td>54/F</td>
<td>Breast cancer node involvement</td>
<td>CVPP (6 mo)</td>
<td>PR</td>
<td>65</td>
<td>5</td>
<td>46,XX,t(7;9)(q22;?),t(21;9)(q22;?),t(12;9)(q22;?)</td>
<td>36/11</td>
<td>Blood 13%</td>
</tr>
<tr>
<td>58b</td>
<td>49/M</td>
<td>Non-Hodgkin's DH/HIA</td>
<td>X-rays mantle</td>
<td>CR</td>
<td>51</td>
<td>62</td>
<td>46,XY,−7,+mar</td>
<td>22/0</td>
<td>Blood 1%</td>
</tr>
</tbody>
</table>

* Examined in preleukemic phase.
* NS, nodular sclerosis; MC, mixed cellularity; MOPP, mechloretamine + vincristine + procarbazine + prednisone; 4-epi-Adm, 4-epi-doxorubicin.

Fig. 1. Hybridization of mutation-specific oligomers to in vitro amplified DNA samples obtained from mononuclear cells of bone marrow or peripheral blood of nine overt leukemias and four preleukemias related to treatment with alkylating agents. 5 ng of DNA were spotted to Gene Screen Plus and hybridized to oligomers representing wild type and mutation-specific codon 13 N-ras. A point mutation in N-ras codon 13 was detected in case no 80 (arrow).
phocytic leukemia related to therapy with alkylating agents. However, the phenomenon is not common, as it was observed in only one out of nine cases examined. No abnormality was observed in four patients with therapy-related preleukemia. The results seem to rule out point mutations in the Ha-ras, Ki-ras, and N-ras protooncogenes as causally related to the high risk of acute nonlymphocytic leukemia following treatment with alkylating agents. Nor do the c-ras mutations appear to represent a general and inevitable step in the pathogenesis of acute nonlymphocytic leukemia, when our results are evaluated together with the results from two recently published series of patients with acute nonlymphocytic leukemia de novo (31, 32). The dubious role of c-ras mutations in the pathogenesis of acute nonlymphocytic leukemia is further emphasized by the fact that such mutations are sometimes detected in only a small fraction of the leukemic cells (32), and if observed in the initial leukemia, c-ras mutations may not be demonstrable at relapse.

With the technique described, increasing the amount of target sequence more than 10^5-fold, the percentage of leukemic blasts present in all samples from our patients with overt leukemia was sufficient to detect any of the point mutations sought. However, in the samples taken from three of our patients with preleukemia or a myelodysplastic syndrome, the negative results might be explained by the marked excess of nonleukemic lymphocytes over leukemic cells. In future studies of patients with preleukemia and myelodysplastic syndromes we suggest that also the granulocytic fraction or unfraccionated nuclear cells from blood or bone marrow should be studied for c-ras mutations.

In a number of studies the protooncogenes c-myc, c-myb, c-fos, c-abl, c-fes, and c-fms have been shown to be expressed in acute nonlymphocytic leukemia (33–36). Except for a few cases, expression of c-myc is modest, as with c-myb, and c-myc expression is directly proportional to the percentage of immature cells in the bone marrow. The c-fos protooncogene seems to be expressed mainly in acute nonlymphocytic leukemia of FAB subtypes M4 and M5 (34) and is supposed to play an important role in the terminal differentiation of monocytes (37). The c-abl expression in acute nonlymphocytic leukemia is generally low as compared to the values in patients with chronic myeloid leukemia, and the expression of c-fes and c-fms is highly variable. Amplification of protooncogenes may occur in acute nonlymphocytic leukemia (38) but is not a common phenomenon, and the transcripts of the various protooncogenes are most often normal. All these results point to a common physiological expression of the various protooncogenes related to proliferation or differentiation. Apart from the mutated ras genes no other protooncogenes have so far been suspected to play a more general pathogenetic role in development of acute nonlymphocytic leukemia.

On the basis of these findings and a recent review challenging simple activation of protooncogenes as a main cause of human cancer (39), one might consider whether other molecular events could lead to the development of therapy-related acute nonlymphocytic leukemia. In recent studies (26, 40) and in the present series, loss of a whole chromosome 5 and/or 7 or various parts of the long arms of these two chromosomes was a very frequent finding. Thus, in these three studies, a -5 or 5q- was observed in 50/119 patients and -7 or 7q- in 79/119 patients. In chromosome 5 the critical region includes bands 5q23-5q32 and in chromosome 7 either bands 7q22-7qter or, as suggested for acute nonlymphocytic leukemia de novo, the much smaller region 7q32-7q44 (41). As these chromosome abnormalities are present even in the early preleukemic stages of therapy-related leukemia with severe refractory cytopenia and an often hypoplastic bone marrow, and as the genes for three important hematopoietic growth factors (42–44) and a growth factor receptor (45) have recently been located to the deleted bands on the long arm of chromosome 5, chromosome 5 deletions have been supposed to play a pathogenetic role in the disease (40). Whether some of the growth factor genes or other genes located to the long arms of chromosomes 5 and 7 could serve in some way as putative repressor genes, by analogy with the hypothesis for retinoblastoma (46–49), Wilm's tumor (50–53) and an increasing number of solid tumors (54–67) showing loss of heterozygosity for specific alleles (Table 3), remains to be determined.

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REFERENCES


Toorchen, I.), and Topai, M. D. Mechanisms of chemical mutagenesis and carcinogenesis: effects on DNA replication of methylation at the guanine position of dGTP. Carcinogenesis (Lond.), 4: 1591-1597, 1983.


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