N-ras Mutations Are Associated With Poor Prognosis and Increased Risk of Leukemia in Myelodysplastic Syndrome


To evaluate the clinical significance of N-ras mutations in the myelodysplastic syndrome (MDS) archival bone marrow samples from 252 patients were studied for the presence of N-ras exon I mutations using polymerase chain reaction amplification and differential oligonucleotide hybridization. Subsequently, clinical information about these patients was obtained and analyzed. Of 220 evaluable patients, 20 (9%) had point mutation of N-ras involving codon 12. Individuals with N-ras mutation had a significantly shorter survival period than those who were N-ras negative (P = .02). An increased risk of acute myelogenous leukemia (AML) was also found in patients with N-ras mutations (P = .005). N-ras mutations were not associated with any French-American-British (FAB) subtype, with the presence of increased myeloblasts, or with chromosomal aberrations in the bone marrow. However, the presence of increased bone marrow blasts was strongly associated with poor survival rate and risk of AML (P < .001 for each). After stratifying for the percentage of blasts, N-ras mutations remained significantly associated with shorter survival period (P = .04) and increased risk of AML (P = .02). Bone marrow cytogenetic abnormalities, particularly when multiple abnormalities were present, were significantly associated with a poor prognosis (P < .001). In conclusion, N-ras mutation, although relatively infrequent in MDS, is associated with short survival period and increased probability of developing AML.

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THE MYELODYSPLASTIC syndrome (MDS) is a heterogeneous group of disorders characterized by peripheral blood cytopenias, trilineage dysplasia of hematopoietic progenitor cells, and an increased risk of developing acute myelogenous leukemia (AML). Individuals with MDS have a high incidence of infection and bleeding resulting from neutropenia and thrombocytopenia, respectively. Several prognostic variables that predict survival and risk of AML in MDS have been identified. Increased bone marrow myeloblasts, the presence of peripheral blood myeloblasts, and the degree of neutropenia or thrombocytopenia have been found to be significant prognostic features at presentation. Cytogenetic analyses have also been shown to be of prognostic value in MDS. Although an abnormal karyotype has not uniformly been found to be associated with a poor survival rate, the presence of multiple chromosomal abnormalities has been linked to a poor prognosis. In addition, chromosomal alterations related to exposure to alkylating agents, especially 5q-, -7, or 7q-, have been associated with decreased rate of survival. Patients with MDS are at increased risk of evolving AML when they have either increased bone marrow myeloblasts or bone marrow cytogenetic abnormalities, particularly when the chromosomal alterations are complex. Although these prognostic features may help to predict the outcome in MDS patients, they do not explain the diversity of clinical behaviors at the molecular level within this group of disorders.

The ras family of genes encode 21-Kd guanosine triphosphate (GTP)-binding proteins that reside on the inner surface of the cell membrane and that appear to play a role in signal transduction from membrane receptors. The ras genes acquire transforming capabilities in association with point mutation of a single nucleotide within the region encoding the GTP-binding portion of the protein. Mutations of ras genes have been found in a wide variety of human malignancies. Hematologic malignancies have been found to preferentially harbor point mutations of the N-ras gene. Various studies have found N-ras mutations in approximately 30% of AML cases and in 5% to 30% of patients with MDS. Because N-ras mutation likely plays a role in the pathogenesis of a distinct subset of MDS cases, we hypothesized that point mutations of N-ras might be associated with specific clinical features in these patients. Therefore, we retrospectively analyzed a large group of MDS patients to determine the effects of N-ras mutation on risk of evolving AML and on prognosis.

MATERIALS AND METHODS

Patient samples. Archival bone marrow biopsy specimens from patients presenting to Mayo Clinic with the diagnosis of MDS between January 1985 and December 1986 were studied. Clinical information about each patient was obtained by chart review. Patients were treated exclusively with supportive care before the development of AML. Bone marrow aspirate and biopsy specimen slides were reviewed by the authors (R.V.P. and O.L.) and classified into MDS subtypes according to French-American-British (FAB) crite-
Cytogenetic analyses were performed using GTG- or QFQ-banding techniques, as described. Bone marrow biopsy samples from 252 patients were evaluated at the University of California at Los Angeles for the presence of N-ras mutations without access to either clinical or pathologic data. Subsequent evaluation of this data showed that 8 patients had no clinical information available, 4 patients had a diagnosis other than MDS, and 3 patients were in a pediatric age range (age <16 years). Data from these individuals were excluded from statistical analysis, leaving clinical information from 237 patients for further analysis. Samples from 7 of these patients were lost and 10 failed to amplify; thus, the status of the N-ras gene was evaluable in only 220 patients. Twenty-seven patients were seen only at the time of diagnosis and additional patients were lost to follow-up at various times after their initial visit. Clinical information about these patients was analyzed until the time of their censorship. Complete survival data (i.e., whether the patient was alive or had died by the end of the period) was available for 162 patients at 1 year, 145 patients at 2 years, and 115 patients at 4 years after diagnosis.

DNA extraction. DNA was isolated from paraffin-embedded bone marrow samples as described. Biopsy specimens were excised from paraffin blocks, then pulverized with a mortar and pestle embedded in dry ice. Samples were suspended in buffer (500 mmol/L Tris, pH 9.0, 20 mmol/L EDTA, 10 mmol/L NaCl, 1% sodium dodecyl sulfate [SDS]), digested with proteinase K (1 mg/mL), extensively extracted with phenol/chloroform, and precipitated with ethanol.

DNA amplification for N-ras by polymerase chain reaction (PCR) and evaluation by differential oligonucleotide hybridization. DNA samples were amplified for exon 1 of N-ras by PCR as described. DNA (50 ng) from patient samples, human peripheral blood lymphocytes (negative control), Molt-4 cells (N-ras codon 12 position 1 mutant), PA-1 cells (N-ras codon 12 position 12), or patient samples previously shown to harbor an N-ras codon 13 position 1 or 2 mutation was subjected to PCR amplification. The DNA was added to PCR reaction solution (25 μL) containing recombinant Taq polymerase (1 U), two synthetic 20-mer oligonucleotide primers (400 μmol/L of each), Tris, pH 8.8 (10 mmol/L), KCl (50 mmol/L), and MgCl₂ (2.5 mmol/L). Appropriate precautions were taken to prevent and detect contamination of reagents by exogenous DNA, as described. Thirty-five cycles of exponential denaturation (at 93°C), annealing (at 50°C), and extension (at 72°C) were performed using a DNA thermal cycler. Amplified product (10 μL) was electrophoresed on Nusieve/agarose gels (3%/1%; FMC Bioproducts, Rockland, ME) and transferred to nylon membrane (Zetaprobe; Bio-Rad, Richmond, CA) with NaOH (0.4 mol/L). Filters were rinsed in 2X SSPE and air dried.

Filters were prehybridized for 1 hour at 63°C in 5X SSPE, 5X Denhardt's solution, 100 mg/mL denatured sonicated salmon sperm DNA, 10 mmol/L EDTA, and 1% SDS. Hybridization was performed for 2 hours under the same conditions in the presence of 20-mer oligonucleotide probes (2 × 10⁶ cpm/mL hybridization solution) end-labeled with [γ-³²P]adenosine triphosphate. For the detection of mutations, mixtures of oligonucleotide probes complimentary to each of all possible activating base pair substitutions at positions 1 and 2 of either codons 12 or 13 of N-ras were used (probes illustrated in Padua et al). A probe of normal sequence was used to ensure that equivalent quantities of amplified DNA were used for all samples. After hybridization, high-stringency rinses were performed in 5X SSPE with 0.1% SDS for 5 minutes at 63°C. Filters were subjected to autoradiography with Kodak X-AR 5 film at ~70°C with intensifying screens (Eastman Kodak, Rochester, NY). Before rehybridization, bound probe was removed by rinsing at 70°C for 20 minutes in 0.1X SSPE. Samples were considered positive for N-ras mutation only if the results were reproducible after reamplification and repeat hybridization.

Statistical analysis. Variables at the time of diagnosis were age, sex, bone marrow cytogenetics (normal, single chromosomal abnormality, or multiple abnormalities), the presence or absence of N-ras mutations, and diagnosis (FAB subtype). Bone marrow blasts initially were summarized by a ternary variable, with FAB subtypes refractory anemia (RA) and refractory anemia with ring sideroblasts (RARS) coded as less than 5% blasts, subtypes refractory anemia with excess blasts (RAEB) and chronic myelomonocytic leukemia (CMML) coded as 5% to 20% blasts, and RAEB in transformation (RAEBT) as greater than 20% blasts. Because no difference in survival or risk of AML was observed between patients with 5% to 20% blasts and those with 20% to 30% blasts, these two strata were combined in all analyses. When used as a categorical variable, age was divided into three levels: less than 60 years of age (the 25th percentile); 60 to 76 years of age; and more than 76 years of age (the 75th percentile). Pairwise associations between all variables at the time of diagnosis were assessed by two-way contingency tables. A three-way association among bone marrow cytogenetics, blasts, and N-ras was tested by a log-linear model using BMDP statistical software program 4F. Survival curves for time until death ("overall survival") and time until transformation to AML ("AML-free survival") were estimated by the Kaplan and Meier product-limit method using BMDP program 1L (BMDP Statistical Software, Inc, Los Angeles, CA). The log-rank test, with or without stratification on blasts, was used for univariate comparisons of survival curves between groups. The Cox proportional hazards model (BMDP program 2L) was used for simultaneously testing the influence of all covariates on overall survival or on AML-free survival. Only patients who had complete data for all covariates were analyzed using the Cox model.

RESULTS

N-ras mutations and relationship to other variables. N-ras mutations were identified in bone marrow DNA from 20 of 220 patients (9%). All mutations involved codon 12 at either position 1 or 2; no codon 13 mutations were found (Fig 1). χ² analysis was used to evaluate possible associations between N-ras mutation and other prognostic variables at diagnosis. N-ras mutations occurred with a similar frequency in patients of each FAB subtype (Table 1). No statistical association was found between N-ras mutation and increased bone marrow blasts, chromosomal abnormalities, and age or sex of the patient. Patients with N-ras mutations at either position 1 or position 2 did not differ significantly with respect to any clinical variables. A significant correlation was found between increased bone marrow blasts and chromosomal abnormalities, both single and complex (P < .001). Patients with the FAB subtypes RAEB and RAEBT were more likely to have bone marrow chromosomal abnormalities than those with fewer than 5% bone marrow myeloblasts (Table 2). Although individuals with CMML frequently had increased bone marrow myeloblasts, they had no higher frequency of chromosomal abnormalities than did patients with fewer than 5% bone marrow blasts. In a test of three-way associations, N-ras mutation remained independent of bone marrow blasts and/or chro-
mosomal abnormalities. None of the remaining variables were significantly associated with each other by two-way contingency tables.

Prognostic value of N-ras mutation and other variables. The impact of N-ras mutations on survival was evaluated using product-limit survival analysis and the log-rank test. Survival was significantly shorter in patients with N-ras mutations than in patients without these abnormalities \( (P = .02) \). The median survival period \( (\pm SE) \) of MDS patients with N-ras mutations was 19 ± 7 months compared with 39 ± 14 months for patients without N-ras mutations (Fig 2A). Survival was similar for each type of N-ras codon 12 mutation.

The prognostic value of N-ras mutations was compared with that of other variables previously identified as predictors of survival in MDS (Table 3). The presence of increased bone marrow blasts was a significant predictor of early death \( (P < .001) \). Individuals with ≥5% blasts had a median survival of 17 ± 3 months, whereas those with less than 5% blasts experienced a median survival of 73 ± 7 months (Fig 2B). Bone marrow chromosomal abnormalities, particularly when multiple abnormalities were present, were associated with a significantly shorter survival \( (P < .001) \). Patients with multiple cytogenetic abnormalities had a median survival of 9 ± 4 months; those with a single chromosomal defect had a median survival of 23 ± 4 months; and patients with normal chromosomes had a median survival of 63 ± 18 months (Fig 2C). Neither age nor sex were related to survival duration in our patient population.

To determine which prognostic variables were independent predictors of survival, patients were first stratified into high and low risk groups based on the percentage of bone

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<th>Table 1. Frequency of N-ras Mutations by FAB Subtype</th>
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<th>Table 2. Frequency of Chromosomal Abnormalities by FAB Subtype</th>
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Percentages in parentheses.
marrow blasts. After stratification, the presence of N-ras mutations remained a significant predictor of survival (P = .04), suggesting that these two variables are independent risk factors for early death in MDS patients. When chromosome results were stratified for blasts, the presence of single chromosomal abnormalities no longer predicted a less favorable outcome than the presence of a normal karyotype (Fig 2D). However, patients with complex chromosomal abnormalities remained at significantly increased risk of early mortality than those with either a normal karyotype or a single abnormality (P < .001). These data suggest that, although the percentage of bone marrow blasts is an important predictor of survival in MDS, knowledge about the presence of either N-ras mutations or multiple chromosomal abnormalities provides additional independent prognostic information in MDS patients.

To further quantify the influence of the prognostic variables on survival, a proportional hazards model was used with age, sex, percentage of bone marrow blasts, presence or absence of multiple chromosomal abnormalities, and presence or absence of N-ras mutations as covariates. Increased marrow blasts were associated with a 3.4-fold increase in the risk of death (Fig 3). After stratifying on blasts, the presence of multiple chromosomal abnormalities was associated with a 4.6-fold increase, and the presence of N-ras mutation was associated with a 1.6-fold increase in probability of death. These estimates were similar to the risk estimates in the model without stratifying on blasts. The wide 95% confidence interval for N-ras mutation includes the value 1.0 (ie, no change in risk of death), despite the significant association noted in the univariate log-rank test. This discrepancy may be due, in part, to the fewer number of patients (191) for whom complete data on all covariates were available for evaluation by the proportional hazards model.

N-ras mutation and evolution of AML. Complete data (ie, whether the patient had evolved to AML and/or was still alive at the end of the period) were available for 125 patients at 1 year, for 94 patients at 2 years, and for 56 patients at 4 years after diagnosis. The cumulative number of patients developing AML was 14, 25, and 28 after 1, 2, and 4 years, respectively. Variables at presentation were analyzed for their ability to predict the risk of developing AML. Patients with N-ras mutations had a significantly increased risk for developing AML compared with those without detectable mutations (P = .005; Fig 4A). The frequency of AML was similar for each type of N-ras codon 12 mutation. The presence of increased bone marrow blasts was related to an elevated risk of AML (P < .001; Fig 4B). After stratifying for the percentage of bone marrow myeloblasts, the presence of N-ras mutations remained a significant predictor of AML risk (P = .02). Multiple chromosomal abnormalities had only a borderline association with increased risk of leukemic evolution (P = .09); however, after stratifying for the percentage of marrow blasts the association was stronger (P = .03). Age at diagnosis did not predict leukemic evolution (P = .28), but, unexpectedly, male sex was associated with an increased risk of AML (P = .01). Using the proportional hazards model, increased bone marrow blasts were associated with an 8.4-fold increase in the risk of leukemic evolution (Fig 3). After stratifying on blasts, the estimated proportional increase in the risk of AML in patients with multiple bone marrow chromosomal abnormalities or N-ras mutation was 5.2 or 2.4, respectively (Fig 3). The relative risk of AML associated with male sex was 4.5 (95% confidence interval, 1.3 to 15.3).

DISCUSSION

Several studies have evaluated the frequency of N-ras mutations in MDS, with varied results (Table 4). We identified N-ras exon 1 mutations in 9% of 220 MDS patients using differential oligonucleotide hybridization. This percentage is similar to the average frequency of N-ras mutations identified by previous studies (Table 4). Variations in mutation frequencies among reported data may reflect the heterogeneous nature of the disease and the relatively small sample sizes evaluated at each institution. We did not investigate the frequency on N-ras exon II, K-ras, or H-ras mutations in MDS. Previous work suggests N-ras exon II mutations are much less frequent than exon I mutations in this disorder. Of 252 MDS samples examined for N-ras exon II, only 6 mutations (2%) were reported.20,23-25,27,28,31 H-ras mutations are also uncommon in MDS; only 3 mutations were found in 128 specimens studied23,25,28. Interestingly, all H-ras mutations occurred in patients with other ras gene mutations. Mutations of K-ras occur approximately half as frequently as N-ras mutations in MDS. A total of 12 K-ras mutations, compared with 22 N-ras exon I mutations, were found in 128 patient samples evaluated in several studies.23-25,28 Therefore, by studying solely N-ras exon I mutations, we probably identified the majority of ras mutations in our patient population, but may have overlooked a lesser number of patients with other ras mutations, predominantly involving K-ras. We found N-ras mutations with approximately equal frequency in all subtypes of MDS (Table 1). This contrasts with two earlier studies that reported a particularly high frequency of ras mutations in the CMMIL subclass of MDS.23,28 Because these studies found that ras mutations in CMMIL frequently involved N-ras exon II and K-ras, we may have underestimated the number of ras mutations in this MDS subgroup.

The survival period for patients with N-ras mutations was significantly shorter than for those without mutations (P = .02). Although the percentage of bone marrow myeloblasts was more significantly associated with poor outcome than N-ras mutation, the presence of N-ras mutation remained significant after stratifying the patients into high and low blast populations (P = .04). In addition, the presence of N-ras mutation was not significantly associated with either the percentage of bone marrow blasts or the presence of bone marrow cytogenetic abnormalities. These results suggest that N-ras mutation is an independent predictor of survival duration in MDS patients.

N-ras mutation also was significantly associated with an increased risk of AML (P = .005). Within 2 years of follow-up, 70% of patients with N-ras mutations evolve into AML compared with less than 20% of N-ras-negative patients.
Fig 2. Kaplan-Meier survival plots according to variable subgroups: (A) Survival of patients with \( n = 19 \) initially at risk or without \( n = 174 \) \( N\)-ras mutations. (B) Survival of patients with less than 5% \( n = 113 \) or ≥5% \( n = 96 \) bone marrow blasts.

(Fig 4A). These results are similar to data reported previously for a small study of 27 MDS patients; 73% of \( ras \) mutation-positive patients developed AML compared with 19% of \( ras \) mutation-negative individuals. \( N\)-ras mutation also may be associated with an increased risk of leukemic evolution during childhood MDS. Although the presence of increased bone marrow myeloblasts was the most significant predictor of AML risk \( (P < .001) \), after stratification on this variable \( N\)-ras mutation remained a significant risk factor for leukemic progression \( (P = .02) \). Therefore, \( N\)-ras mutation appears to convey an elevated risk of leukemic evolution in MDS patients. Mutation of the \( N\)-ras gene may
also affect the biology of this disease. Transfection of various cell types with mutant ras genes has been shown to stimulate secretion of interleukin-3, granulocyte, and granulocyte-macrophage colony-stimulating factors. Because these cytokines can be growth factors for myeloid leukemia cells, the acquisition of a mutated ras gene by a myeloid progenitor may lead to autonomous growth through an autocrine mechanism.

The presence of increased bone marrow blasts appears to have the strongest association with poor survival and risk of
developing AML. The majority of studies that have evaluated prognostic factors in MDS have also found this variable to be the best predictor of short survival. The presence of increased marrow myeloblasts reflects a severe impairment of hematopoiesis in addition to an increased proclivity to develop AML. This finding is reinforced by the fact that the majority of MDS patients die from complications of cytopenias rather than from leukemic evolution.

Bone marrow cytogenetic abnormalities also were found to affect survival and risk of AML. A progressive decline in survival was associated with accumulation of chromosomal defects: patients with multiple chromosomal abnormalities had the shortest median survival (9 months); those with normal cytogenetics had the longest (63 months); and individuals with a single chromosomal defect had an intermediate median survival (23 months). A similar stepwise association between number of chromosomal abnormalities and increase in risk of death was identified in one previous study. Other series have reported that any abnormal karyotype or that only multiple chromosomal abnormalities were significantly linked to shortened survival. When we stratified the survival data for the percentage of bone marrow blasts, patients with single chromosomal defects were found to have outcomes similar to those without cytogenetic abnormalities. However, the survival period for MDS patients with complex karyotypic abnormalities was significantly shorter than for the other two groups. Univariate analyses showed only a borderline association between multiple chromosomal abnormalities and risk of AML. Nevertheless, the proportional hazards model predicted that the presence of complex cytogenetic abnormalities was associated with a 5.2-fold increased risk of leukemic evolution, albeit with a wide confidence interval. Previous studies have reported that the presence of any cytogenetic abnormality or only complex chromosomal changes was significantly associated with increased risk of AML.

A significant association between cytogenetic abnormalities and increased bone marrow myeloblasts was identified by $\chi^2$ analysis. The statistical relationship between these variables suggests that as genetic aberrations are accrued by stem cells, they become progressively less capable of differentiating into mature blood cells leading to an accumulation of myeloblasts in the bone marrow. After stratifying on the percent of bone marrow blasts, multiple chromosomal abnormalities were found to be significantly associated with short survival and risk of AML, suggesting that, although these variables are related, complex chromosomal abnormalities provide additional prognostic information in MDS patients. Apparently, in addition to hindering normal differentiation, accumulated chromosomal defects affect the function of either the dysplastic clone or its progeny.

Patient age was not found to affect either survival or risk of leukemic evolution. Although gender did not influence survival, male sex was significantly associated with an increased rate of developing AML. This risk was independent of all other variables, and the explanation for this relationship is unclear.

Although the data were subject to a moderate amount of censoring due to loss of patient follow-up over time, it is unlikely that the censoring was related to the variables examined or to patient outcome. Because our results regarding the influence of bone marrow blasts and chromosomal abnormalities on survival and AML risk agree with those of previously published studies, this claim is strengthened. Although this indirectly supports our evidence that N-ras mu-
mutation decreases survival duration and increases the risk of developing AML, the magnitude of these effects relative to other factors is not completely clear. Based on the relative risks estimated using the proportional hazards model, the impact of N-ras mutation on survival or evolution to AML may be smaller than that caused by the presence of increased bone marrow blasts or complex chromosomal abnormalities. However, because of censoring and additional loss of sample size due to missing covariate data for some patients, the confidence intervals for these regression estimates are quite wide, and the true rank order among the three factors is uncertain.

Fig 4. Kaplan-Meier plots of AML-free survival according to variable subgroups: (A) patients with (n = 19 initially at risk) or without (n = 174) N-ras mutations; (B) patients with less than 5% (n = 113) or ≥5% (n = 96) bone marrow blasts.
In summary, N-ras gene mutation was associated with an increased rate of AML evolution and early death in MDS patients. These associations were independent of the percentage of bone marrow myeloblasts and the presence of cytogenetic abnormalities. Identification of a subset of MDS patients with N-ras mutations may be the first step in developing a new classification scheme in which clinical behavior of MDS patients can be predicted on the basis of specific genetic mechanisms rather than by secondary morphologic abnormalities observed in the bone marrow.

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Table 4. Frequency of N-ras Mutations in MDS

<table>
<thead>
<tr>
<th>Investigators</th>
<th>N-ras Mutations/ Patients Studied (%)</th>
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<tr>
<td>Janssen et al</td>
<td>1/15 (7)</td>
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<td>Lyons et al</td>
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<td>Padua et al</td>
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<td>Bar Eli et al</td>
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<td>Mano et al</td>
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<td>Yunis et al</td>
<td>9/27 (33)</td>
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<td>Browett and Norton</td>
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<td>van Kamp et al</td>
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<td>Total</td>
<td>32/273 (12)</td>
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<td>This study</td>
<td>20/220 (9)</td>
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