

DECISION MAKING AND PROBLEM SOLVING



A one-mutation mathematical model can explain the age incidence of acute myeloid leukemia with mutated nucleophosmin (*NPM1*)

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ABSTRACT

Acute myeloid leukemia (AML) with mutated *NPM1* gene and aberrant cytoplasmic expression of nucleophosmin (NPMc⁺ AML) shows distinctive biological and clinical features. Experimental evidence of the oncogenic potential of the nucleophosmin mutant is, however, still lacking, and it is unclear whether other genetic lesion(s), e.g. *FLT3* internal tandem duplication (ITD), cooperate with *NPM1* mutations in AML development. An analysis of age-specific incidence, together with mathematical modeling of AML epidemiology, can help to uncover the number of genetic events needed to cause leukemia. We collected data on age at diagnosis of AML patients from five European Centers in Germany, The Netherlands and Italy, and determined the age-specific incidence of AML with mutated *NPM1* (a total of 1,444 cases) for each country. Linear regression of the curves representing age-specific rates of diagnosis per year showed similar slopes of about 4 on a double logarithmic scale. We then adapted a previously designed mathematical model of hematopoietic tumorigenesis to analyze the age incidence of AML with mutated *NPM1* and found that a one-mutation model can explain the incidence curve of this leukemia entity. This model fits with the hypothesis that NPMc⁺ AML arises from an *NPM1* mutation with haploinsufficiency of the wild-type *NPM1* allele.

Key words: acute myeloid leukemia, nucleophosmin, mutation.

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Introduction

The nucleophosmin (*NPM1*) gene, which encodes a nucleolar multifunctional protein, is frequently translocated or mutated in hematological malignancies.^{1,2} Mutation of *NPM1* is one of the most common genetic alterations in adult acute myeloid leukemia (AML), occurring in about one-third of patients and accounting for 50-60% of all AML cases with normal karyotype.³ Since *NPM1* mutations were first discovered in AML in 2005,¹ about 40 mutation variants have been identified.³ Despite molecular heterogeneity, all variants lead to common changes at the C-terminus of the NPM1 protein⁴ which cause an increased nuclear export of the nucleophosmin leukemic mutant and its aberrant accumulation in the cytoplasm of leukemic cells;^{4,6} hence the term NPMc+ (cytoplasmic-positive) AML.^{1,3} AML with mutated NPM1 shows distinctive biological and clinical features,⁵ including a unique gene expression profile,^{7,8} a distinct microRNA signature,⁹ frequent CD34-negativity (more than 95% of cases),^{1,3} increased incidence of FLT3-ITD mutations (about 40% of cases),¹ good response to induction therapy¹ and a favourable prognosis (in the absence of *FLT3-ITD*).¹⁰⁻¹⁵ These findings strongly suggest that AML with mutated *NPM1* represents a new disease entity. Experimental evidence of the oncogenic potential of the nucleophosmin mutant is, however, still lacking, and it is unclear whether other genetic lesion(s), such as *FLT3-ITD*, cooperate with *NPM1* mutations in generating the leukemic phenotype. The multi-step theory of carcinogenesis was conceived after mathematical modeling demonstrated that the increasing cancer incidence with age can be explained by several stochastic events needed for tumorigenesis.¹⁶⁻¹⁹ A recently developed population genetics model²⁰ was used to study the age-specific incidence of chronic myeloid leukemia and found that the data are consistent with the hypothesis that the BCR-ABL fusion oncogene alone is sufficient to cause the chronic phase of the disease. Later on, Vickers demonstrated that the age of onset of polycythemia vera is in accordance with the assumption of a single rate-limiting mutation and a small number of stem cell divisions per year.²¹

To investigate the age-specific incidence of AML with mutated *NPM1*, we adapted the one-mutation model that was originally designed to describe chronic myeloid leukemia age distribution.²⁰ The model fits the NPMc+ AML age-specific incidence curve assuming plausible parameter values, supporting the hypothesis that a single genetic event, the *NPM1* mutation, is sufficient to cause leukemia. The role of *NPM1* mutations in AML development is discussed in the light of these findings.

Design and Methods

Patients

National registry-based AML incidence data with details of *NPM1* mutation status are not available.

Therefore, we collected data sets at five major European Institutions involved in the diagnosis and treatment of AML patients: (i) the Laboratory of Cytogenetic and Molecular Diagnostics, University Hospital Ulm, representing the German-Austrian AML study Group (AMLSCG); (ii) the Laboratory of Hemopathology, Institute of Hematology, University of Perugia, representing the Gruppo Italiano Malattie Ematologiche dell'Adulto (GIMEMA); (iii) the Laboratory for Molecular Diagnostics, University Hospital Carl Gustav Carus, Dresden, Germany, representing the Deutsche Studieninitiative Leukämie (DSIL); (iv) the Munich Leukemia Laboratory (MLL), Munich, Germany; and (v) the Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands.

A total of 1,444 AML patients (age range: 20-59; median 47) carrying a mutated *NPM1* gene were included in this study (n=476 from AMLSCG; n=354 from GIMEMA; n=251 from DSIL; n=223 from MLL; and n=140 from The Netherlands). Exclusion criteria were: i) patients under 20 years of age because few cases were available, due to the low frequency of *NPM1* mutations in this age group²²; and ii) patients over 59 years of age who are often treated in local hospitals. Consequently, those patients referred to major institutions for diagnosis and treatment may not be representative of the population of AML patients in this age group.

Information on *FLT3* status was available in 1,386/1,444 AML patients with mutated *NPM1* (96%). *FLT3-ITD* was detected in 553/1,386 cases (40%). For analysis, the 1,444 *NPM1*-mutated AML patients were stratified in 5-year age classes.

For this study, we assume that the mutational event needed to develop NPMc+ AML occurs independently of local exposure to environmental leukemogenic factors and that the age specific rates of *NPM1*-mutated AML patients 20-59 years in age reflect those of the general population in the three European countries included in the study.

Modeling age specific incidence

The mathematical model by Michor *et al.*²⁰ was adapted to analyze the AML incidence data. Our model is based on the following considerations: (i) we consider a population of N hematopoietic stem cells. Initially, all cells are wild type and proliferate according to a stochastic process known as the Moran model:²³ every τ days, a cell is chosen at random proportional to fitness to divide; its offspring replaces another randomly chosen cell. The population size is strictly constant. (ii) a wild-type cell gives rise to a mutated cell at rate u per cell division. A mutated cell has a relative growth rate (fitness) of r . If $r=1$, the mutation is neutral as compared to wild type cells; if $r<1$, the mutant is disadvantageous, and if $r>1$, the mutant has a proliferation advantage over the wild-type cell. We assume that an *NPM1* mutation confers a fitness advantage to the cell, $r>1$. (iii) Our model adheres to standard Moran process until a surviving mutant cell appears; thereafter, clonal growth is initiated that continues until the mutated cell population reaches population size \bar{N} . Unlike the model designed by Michor *et al.*,²⁰ which assumes a constant population

size of N cells, our model allows the mutant clone to expand until a maximal size, \bar{N} . This change is intended to account for the marked expansion of the initial cell compartment which is peculiar to AML. (iv) The AML detection rate is proportional to the number of mutated cells present; if there are N_m mutated cells, the rate of diagnosis is $q N_m$. From assumptions (i) and (ii) it follows that the waiting time for the first successful (=surviving) mutation has a negative exponential distribution, $b=Nu(1-1/r)$. Let a be the time since the occurrence of the first surviving mutation. Then assumption (3) states that the number of mutated cells, N_m , grows according to

$$\dot{N}_m(a) = cN_m(a)(1-N_m(a)/\bar{N})$$

where $c=(r-1)/\tau$ and $N_m(0)=1/(1-1/r)$. To account for the significant expansion of the mutated clone, we assume $\bar{N} \gg N$. Finally, if (q) is the proportionality constant between the rate of detection and the number of mutated cells (assumption iv), then the probability of diagnosis²⁰ at time t is given by

(1)

$$P(t) = \int_0^t \left[1 - \left(1 + \frac{e^{cz} - 1}{\bar{N}(1-1/r)} \right)^{-q\bar{N}/c} \right] e^{-b(t-z)} b dz$$

We compared the predictions of equation (1) with the direct computer simulation of the stochastic process. The simulation is performed by first determining the time at which the first surviving mutated cell arises in a population of N wild type cells; this time follows a negative exponential distribution with mean $1/b$. Once such a cell has emerged, the branching process of clonal expansion is simulated by choosing a cell for reproduction or for death at random at each time step. The probability that the number of wild type cells, N , increases by one is given by

(2a)

$$\Pr[(N, N_m) \rightarrow (N+1, N_m)] = N(1-u)/\Gamma$$

where $\Gamma = (1+d)N + (r+d)N_m$. Here d denotes the death rate of both wild type and mutated cells. The probability that the number of mutated cells, N_m , increases by one is given by

(2b)

$$\Pr[(N, N_m) \rightarrow (N, N_m+1)] = (Nu + rN_m)/\Gamma$$

The probabilities that the numbers of wild type and mutated cells decrease by one are respectively given by

(2c)

$$\Pr[(N, N_m) \rightarrow (N-1, N_m)] = dN/\Gamma$$

(2d)

$$\Pr[(N, N_m) \rightarrow (N, N_m-1)] = dN_m/\Gamma$$

A patient is diagnosed at rate qN_m and is entered into the incidence data base of his age class. *Online Supplementary Figure 1* shows the fit of equation (1) and system (2). Under particular circumstances, i.e. when the waiting time for the first successful mutation is long and clonal expansion occurs fast and reaches large cell numbers, the incidence data can be a kinked curve. A more detailed mathematical investigation of such situations is forthcoming (*Michor F. et al., in preparation*) but will not be discussed here since the experimentally determined incidence data is a straight line on a doubly logarithmic plot.

Finally, we compared equation (1) with the experimental data, which allowed us to quantify AML-specific parameters.

Statistical analysis

The χ^2 test ($\alpha < 0.05$) was used to assess independence of the age distribution of cases by centre of diagnosis. The likelihood ratio test, comparing a Poisson regression model including age, country, and age x country interaction terms with the nested model without the interaction term was performed to evaluate dependence of age-specific *NPM1*-mutated AML rates on the country.

Results

Age-specific rates of AML with mutated *NPM1* are similar in different countries

First, we determined whether age-specific incidence curves of AML with *NPM1* mutations were comparable in Italy, Germany and The Netherlands. The AML cases registered by each center do not provide a precise estimate of incidence, since the population that is referred to each study center for diagnosis cannot be identified. However, it is important to note that the slope of the incidence curve is needed for our purpose, not population-based incidence figures. Therefore, population data from the U.S. Census Bureau website (<http://www.census.gov/ipc/www/idb>, accessed on February 12, 2008) were used to obtain demographic data (person-years) for each country. In each country the number of AML cases was stratified into age-classes. The cases in each age-class were divided by the total population in that age-class, which provided the age-specific rate of diagnoses per year per million inhabitants.

Chi-square testing of the age distribution of cases on center of diagnosis was not significant ($p=0.48$) indicating that, although absolute incidence levels vary because they reflect the percentage of the general population that is covered by participating centers, number of cases by age class does not differ among study centers (Table 1). The likelihood ratio test comparing the Poisson model which includes a country x age class interaction with the simpler model without the interaction term (*Online Supplementary Table 1*) was non-significant ($p=0.85$). Together, these findings provide evidence that AML data from the three countries are comparable. In particular, AML incidence curves analyzed via linear regression all showed a slope of about 4 on a log-log scale (Figure 1).

The one-mutation model fits the incidence curve of AML with mutated NPM1

We next adapted the one-mutation mathematical model that was originally designed to describe chronic myeloid leukemia epidemiology²⁰ to investigate age-specific incidence data in AML with *NPM1* mutations. The model provided adequate data fitting and generated slopes similar to real age-specific incidence curves from patients (Figure 2) from Germany, The Netherlands, and Italy. The corresponding χ^2 and P-values for the three countries were 0.02027 ($p=0.9899$), 0.00862 ($p=0.9956$), and 0.15275 ($p=0.9264$). The fitting procedure provided estimates of the parameters in each country (Table 2) generating numbers that are biologically plausible (see below).

The initial haematopoietic stem cell (HSC) compartment was quantified as 1.03×10^4 - 1.14×10^4 . This fits with experimental findings²⁴ suggesting that, although humans require more blood cells per lifetime than mice (because of their larger size and longer life expectancy), the total human HSCs number is equivalent to the total number of HSCs in mice, which has been shown to be of about $11,400 \pm 5,400$.²⁴

The maximum number of mutated cells generated by the model was about $\bar{N}=10^{13}$. This number is consistent with the high tumor burden observed in leukemia patients, if one assumes that, under physiological conditions, the amount of human nucleated marrow cells per kg body weight has been calculated to be approximately 2.1×10^{10} (1.5×10^{12} in a subject of 70 kg).²⁵ The relative fitness of mutated cells spanned the range 1.38-1.61. The mean cell generation time (i.e. the time needed for a cell to divide), was between 2.67 and 3 days, which concurs with early experimental findings²⁶ and with clinical data.³ In the *NPM1*-mutated AML case, the rate of cancer detection per mutated cell was found to be in the range of 7.77×10^{-5} - 1.58×10^{-4} days. This implies that the total rate of detection (qN) is in the range of 0.26-1.78, which is higher than previous estimates in chronic myeloid leukemia.²⁰ Leukemic clones are initiated by single *NPM1* mutations occurring at rates ranging from 2.43×10^{-9} to 4.86×10^{-9} days per cell division. Taken together, these estimates imply that for a single individual the waiting time for the appearance of a surviving mutation is on average $1/(Nu(1-1/r))$, which is about 5532, 9940 and 8779 days for Germany, Netherlands and Italy, respectively.

Table 1. Cumulative incidence of the NPM1-mutated AML in Germany (DE), the Netherlands (NL) and Italy (IT).*

Age class	Cum P (DE)	Cum P (NL)	Cum P (IT)
20-24	8.87×10^7	9.73×10^7	5.92×10^7
25-29	2.55×10^6	2.07×10^6	3.58×10^6
30-34	4.34×10^6	4.36×10^6	6.29×10^6
35-39	8.17×10^6	7.72×10^6	1.10×10^5
40-44	1.35×10^5	1.29×10^5	1.76×10^5
45-49	1.93×10^5	1.84×10^5	2.56×10^5
50-54	3.05×10^5	2.45×10^5	3.72×10^5
55-59	3.81×10^5	3.49×10^5	4.85×10^5

*Corresponding curves have a slope of about 4 on a double logarithmic scale (slope DE=4.038, slope NL=4.043, slope IT=4.332) as seen in Figure 1 (panels A, B and C).

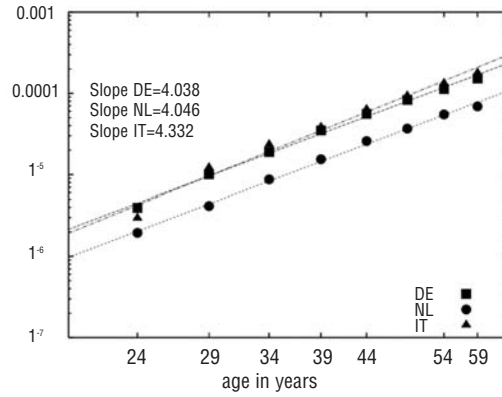


Figure 1. Incidence data for the NPM1⁺ AML in Germany (DE), The Netherlands (NL) and Italy (IT). Linear regression shows a slope of about 4 on a doubly logarithmic scale.

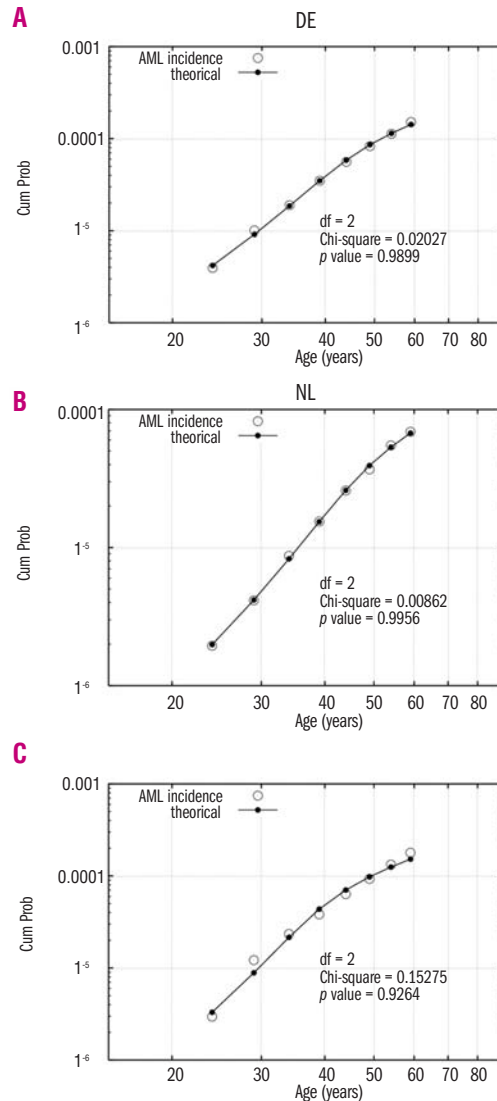


Figure 2 (right). The incidence data can be fit to the one-mutation model assuming plausible parameter values for Germany (A), The Netherlands (B) and Italy (C). χ^2 and corresponding p-values reported as labels in the corresponding figures show the quality of the fit.

Table 2. Parameters of the one-mutation model for all *NPM1* mutated AMLs.

Parameter	Definition	Germany	Netherlands	Italy
r	Relative fitness	1.42	1.38	1.61
τ	Mean cell generation time	2.79	2.67	3.00
q	Rate of cancer detection per mutated cell	1.58×10^{-4}	1.56×10^{-4}	7.77×10^{-5}
N	Standard number of hematopoietic stem cells	1.10×10^4	1.14×10^4	1.03×10^4
u	Mutation probability per cell division	4.86×10^{-9}	2.43×10^{-9}	4.19×10^{-9}
\bar{N}	Maximum number of mutated cells	1.00×10^{13}	1.00×10^{13}	1.00×10^{13}

Value were obtained via minimization of the least squares function in eq. (2).

FLT3* gene status does not influence the age-specific incidence of AML with mutated *NPM1

Internal tandem duplication (ITD) at the *FLT3* gene locus has been implicated as a cooperating genetic alteration in various AML subtypes.^{27,28} Since *FLT3*-ITD frequently associates with *NPM1* mutations¹ and appears to abrogate the favourable prognostic effect of *NPM1* mutations in AML1,^{15,29} we determined whether the age incidence of *NPM1*-mutated AMLs with *FLT3*-ITD differs from cases with wild-type *FLT3*. No significant difference emerged in the slopes of *FLT3*-ITD-positive and -negative AML with mutated *NPM1* (Figure 3). The quality of fit with the model-generated data was adequate and similar to the quality of fit for all AMLs with *NPM1* mutations (Figure 4). The one-mutation model parameters for fitting *FLT3*-ITD positive and *FLT3*-ITD negative AML with mutated *NPM1* are reported in Table 3. The slopes of the three groups (*NPM1* mutated, *NPM1* mutated/*FLT3*-ITD, *NPM1*-mutated/*FLT3* wild-type) are not significantly different according to the Mann-Whitney U test ($p > 0.05$) (Online Supplementary Table 2).

Discussion

In this study, we adapted a one-mutation mathematical model that was originally designed to describe chronic myeloid leukemia epidemiology²⁰ to investigate the age-specific incidence data in AML with mutated *NPM1*. The model fits the *NPM1*^c AML age-specific incidence curve for plausible parameter choices, supporting the hypothesis that a single genetic event, the *NPM1* mutation, is sufficient to cause this type of leukemia. However, evidence derived from *in vitro* functional studies and experimental models are required to confirm or refute this hypothesis.

Our findings add to the body of evidence that the *NPM1* mutation is a founder genetic lesion in *NPM1*^c

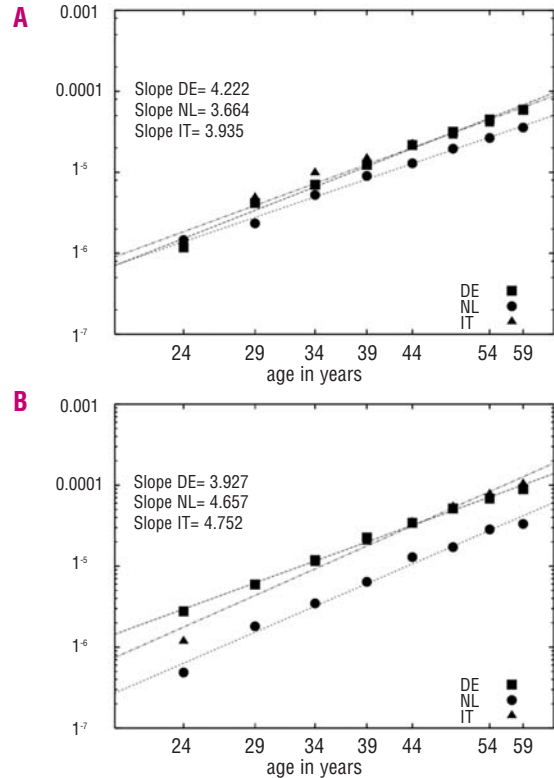


Figure 3. Incidence data for the *NPM1*^c AML bearing *FLT3*-ITD (A) or *FLT3* wild-type for Germany (DE), The Netherlands (NL) and Italy (IT) (B).

AML: i) cytoplasmic mutated nucleophosmin is specific for AML^{1,30,31} and clinically shows close association with AML of *de novo* origin^{1,32-34}; ii) all *NPM1* mutations generate changes at the C-terminus of nucleophosmin protein which appear to maximise nuclear export of *NPM1* leukemic mutants,^{3,35-37} pointing to cytoplasmic dislocation of the mutants as the central event for leukemogenesis; iii) *NPM1* mutations are mutually exclusive with other recurrent genetic abnormalities,^{1,33} with the exception of rare cases in which both *NPM1* and *CEPBA* (or *FLT3*-ITD) mutations are found;¹⁵ iv) they are stable during the course of the disease^{39,40} as the same type of *NPM1* mutation is consistently detected at relapse in medullary and extramedullary sites;⁴⁰ and v) quantitative real-time PCR shows that *NPM1* mutations disappear at complete remission.^{41,42}

The major finding in the present study is that the one-mutation mathematical model can explain the age-specific incidence in *NPM1*^c AML. This hypothesis is in contrast to current concepts in AML development which, like other human cancers, is believed to be a consequence of more than one oncogenic hit.⁴³ Indeed, several animal models of AML clearly point to leukemogenesis as a multi-step process.⁴³ Moreover, *in vitro* findings that the *NPM1* leukemic mutant specifically cooperates with the E1A adenovirus to transform primary MEFs in soft agar⁴⁴ suggest that *NPM1* mutations need to act in close concert with other oncogenic hits. In MEF cells, this mutual cooperation involves the *NPM1* mutant inhibiting the E1A-elicited p19(Arf) induction

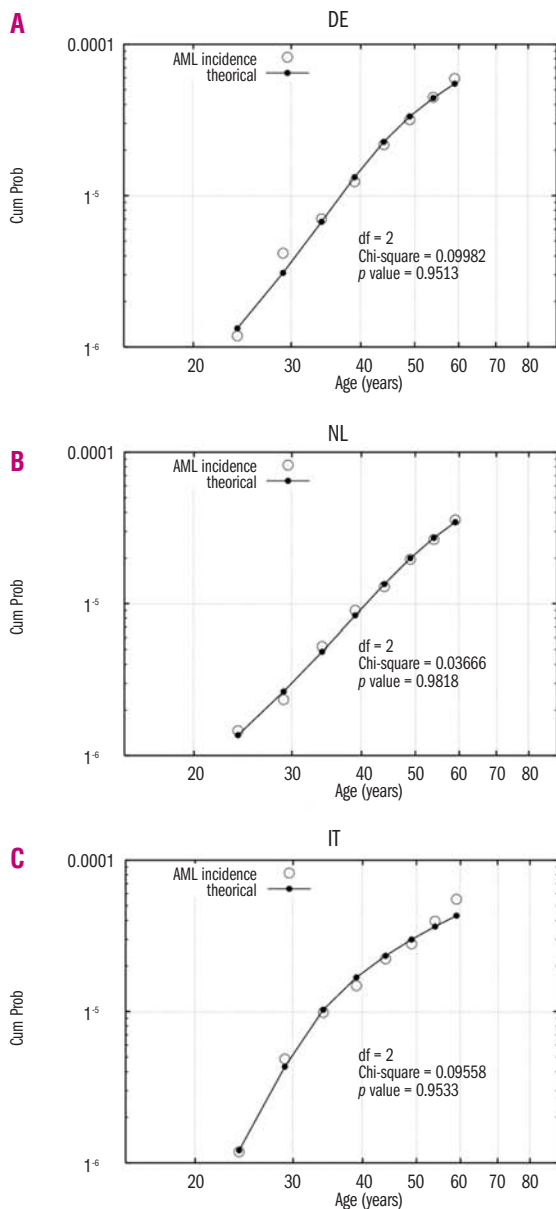


Figure 4. Fitting the incidence data with the one-mutation model (A) leads to plausible parameter values in all cases (NPMc⁺ AML with *FLT3*-ITD or *FLT3* wild-type) for all three countries (Germany, The Netherlands (B) and Italy (C)). χ^2 and corresponding p -values reported as labels in the corresponding figures show the quality of the fit.

and E1A overcoming *NPM1* mutant-induced cellular senescence.⁴⁴ Furthermore, an activating mutation of the *FLT3* gene (*FLT3*-ITD) leading to an internal tandem duplication of the juxtamembrane portion of *FLT3*, a receptor which plays an important role in controlling proliferation and/or survival of hematopoietic progenitors, has been implicated as a cooperating genetic alteration in various AML subtypes.^{27,28} Since *FLT3*-ITD has been detected in about 40% of AML with mutated *NPM1*,¹ it has been suggested that it may play an important role also in this leukemia subtype.

The findings of this paper suggest that the role of

Table 3. Parameters of the one-mutation model for the *NPM1*-mutated/*FLT3*-ITD and *NPM1*-mutated/*FLT3* wild-type (wt) subsets.

Parameter Definition		Germany		Netherlands		Italy	
		ITD	wt	ITD	wt	ITD	wt
N	Standard number of hematopoietic stem cells ($\times 10^4$)	1.00	1.11	1.09	1.11	1.10	1.10
\bar{N}	Maximum number of mutated cells ($\times 10^{12}$)	9.99	10.00	10.00	10.00	9.99	9.99
r	Relative fitness	1.57	1.40	1.34	1.54	1.85	1.62
τ	Mean cell generation time (days)	3.41	2.79	2.87	2.90	2.86	2.90
q	Rate of cancer detection per mutated cell ($\times 10^{-4}$)	1.29	1.84	2.54	0.64	0.32	0.43
u	Mutation probability per cell division ($\times 10^{-9}$)	2.03	2.96	1.52	0.97	0.73	2.39

FLT3-ITD as a cooperative mutation in the pathogenesis of NPMc⁺ AML should be interpreted with caution. In fact, no difference can be detected between the slopes of the age specific incidence of *FLT3*-ITD-positive and -negative NPMc⁺ AML, supporting the view that NPMc⁺ AML is a homogeneous group irrespective of the *FLT3* mutational status. This is consistent with the observation that the unique gene expression profile of AML with mutated *NPM1*, i.e. upregulation of *HOX* genes and downregulation of *CD34*,^{7,8} does not appear to be significantly influenced by the *FLT3* gene status. This is also in keeping with the clinical observation that *FLT3*-ITD can appear or disappear in *NPM1*-mutated AML patients during the course of the disease.³⁹ Moreover, in oncogenic cooperation tests, the *NPM1* leukemic mutant and *FLT3*-ITD did not cooperate to transform mouse embryonic fibroblasts (MEFs).⁴⁴ Hypothetically, *FLT3*-ITD may not be necessary for the development of AML but rather provide a selective advantage for leukemic cells that already harbour the *NPM1* mutation. Unfortunately, there is as yet no experimental mouse model to prove or disprove this hypothesis. However, this interpretation would at least fit with the clinical observation that *FLT3*-ITD appears to abrogate the favorable prognostic impact of *NPM1* mutations,²⁹ suggesting that it may play a role at later stages of NPMc⁺ AML, leading to a more aggressive AML phenotype.

Thus, how can we reconcile the results of our one-mutation mathematical model with current evidence that favor the hypothesis that AML is the result of more than one oncogenic hit?⁴³ One possible explanation is that NPMc⁺ AML arises from the concerted action of an *NPM1* mutation and another leukemogenic event occurring at the same time. Since the *NPM1* mutant has

intrinsic oncogenic properties⁴⁴ and in knock-out mice NPM haploinsufficiency results in a MDS-like syndrome⁴⁵ and in overt leukaemia,⁴⁶ an attractive hypothesis would be that these alterations act together to cause NPMc⁺ AML.^{3,47} Indeed, *NPM1* mutations are associated with haploinsufficiency of wild-type NPM in leukemic cells, since mutations are always monoallelic³ and lead to dislocation of functionally active wild-type NPM from the nucleoli to the cytoplasm through formation of heterodimers with the *NPM1* leukemic mutant.⁴

However, other scenarios cannot be excluded with certainty only on the basis of the mathematical model. *NPM1* and yet undiscovered mutation(s) may act synergistically such that their actions cannot be discerned when investigating incidence data. Moreover, even though *NPM1* mutations may be sufficient to cause leukemia, secondary mutations (e.g. *FLT3-ITD*) could increase the fitness of leukemic cells and/or result in the development of more aggressive AML stages. Finally, it is still possible that cancer incidence data cannot be used to identify the number of genetic changes necessary to cause cancer. Therefore, further experimental studies are warranted to clarify the oncogenic role of *NPM1* mutations and other putative cooperating genetic lesions in NPMc⁺ AML.

Authorship and Disclosures

AL and BF had the original idea, coordinated the whole project and wrote the paper; FC and AC adapted the onemutation mathematical model to the study of AML with mutated NPM1 and contributed to write the manuscript. FS performed the statistical analyses on

incident cases before fitting the one mutation model; RFS collected molecular and clinical data from patients of the AMLSG study and contributed to write the manuscript; SA was involved in designing the GIMEMA study and 18 collecting clinical data from patients; CT performed molecular analyses of patients from DSIL and contributed to write the manuscript; SS performed mutational analysis in patients from the Munich Leukemia Laboratory (MLL) and contributed to write the manuscript; PJMV carried out molecular studies on AML patients from Netherlands and reviewed the manuscript; KD collected molecular and clinical data from patients of AMLSG study and contributed to write the manuscript; MFM recruited patients in the GIMEMA study and reviewed the manuscript; MS designed and coordinated the clinical study (DSIL); JK collected molecular and clinical data from patients of the AMLSG study; AG collected clinical data from patients of the AMLSG study and coordinated the clinical study (AMLSG); MPM and NB performed immunohistochemical studies on the GIMEMA patients; BL recruited patients from Netherlands and reviewed the manuscript; TH coordinated the study of patients from the Munich Leukemia Laboratory (MLL) and contributed to write the manuscript; GE designed and coordinated the clinical study (DSIL); FM designed and coordinated the clinical study (GIMEMA); HD designed and coordinated the clinical study (AMLSG); FM carried out computational simulation studies and contributed to write the manuscript.

The authors reported no potential conflicts of interest.

References

- Falini B, Mecucci C, Tiacci E, Alcalay M, Rosati R, Pasqualucci L, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. *N Engl J Med* 2005;352:254-66.
- Falini B, Nicoletti I, Bolli N, Martelli MP, Liso A, Gorello P, et al. Translocations and mutations involving the nucleophosmin (NPM1) gene in lymphomas and leukemias. *Haematologica* 2007;92:519-32.
- Falini B, Nicoletti I, Martelli MF, Mecucci C. Acute myeloid leukemia carrying cytoplasmic/mutated nucleophosmin (NPMc⁺ AML): biologic and clinical features. *Blood* 2007;109:874-85.
- Falini B, Bolli N, Shan J, Martelli MP, Liso A, Pucciarini A, et al. Both carboxy-terminus NES motif and mutated tryptophan(s) are crucial for aberrant nuclear export of nucleophosmin leukemic mutants in NPMc⁺ AML. *Blood* 2006;107:4514-23.
- Quentmeier H, Martelli MP, Dirks WG, Bolli N, Liso A, Macleod RA, et al. Cell line OCI/AML3 bears exon-12 NPM gene mutation-A and cytoplasmic expression of nucleophosmin. *Leukemia* 2005;19:1760-7.
- Falini B, Martelli MP, Bolli N, Bonasso R, Ghia E, Pallotta MT, et al. Immunohistochemistry predicts nucleophosmin (NPM) mutations in acute myeloid leukemia. *Blood* 2006;108:1999-2005.
- Alcalay M, Tiacci E, Bergomas R, Bigerna B, Venturini E, Minardi SP, et al. Acute myeloid leukemia bearing cytoplasmic nucleophosmin (NPMc⁺ AML) shows a distinct gene expression profile characterized by up-regulation of genes involved in stem-cell maintenance. *Blood* 2005;106:899-902.
- Mullighan CG, Kennedy A, Zhou X, Radtke I, Phillips LA, Shurtleff SA, et al. Pediatric acute myeloid leukemia with NPM1 mutations is characterized by a gene expression profile with dysregulated HOX gene expression distinct from MLL-rearranged leukemias. *Leukemia* 2007;21:2000-9.
- Garzon R, Garofalo M, Martelli MP, Briesewitz R, Wang L, Fernandez-Cymering C, et al. Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin. *Proc Natl Acad Sci USA* 2008;105:3945-50.
- Schnittger S, Schoch C, Kern W, Mecucci C, Tschulik C, Martelli MF, et al. Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. *Blood* 2005;106:3733-9.
- Dohner K, Schlenk RF, Habdank M, Scholl C, Rucker FG, Corbacioglu A, et al. Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. *Blood* 2005;106:3740-6.
- Verhaak RG, Goudswaard CS, van Putten W, Bijl MA, Sanders MA, Hagens W, et al. Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. *Blood* 2005;106:3747-54.
- Thiede C, Koch S, Creutzig E, Studel C, Illmer T, Schaich M, et al. Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia

- (AML). *Blood* 2006;107:4011-20.
14. Gale RE, Green C, Allen C, Mead AJ, Burnett AK, Hills RK, et al. The impact of FLT3 internal tandem duplication mutant level, number, size and interaction with NPM1 mutations in a large cohort of young adult patients with acute myeloid leukemia. *Blood* 2008;111:2776-84.
 15. Schlenk RF, Döhner K, Krauter J, Fröhling S, Corbacioglu A, Bullinger L, et al. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. *N Engl J Med* 2008;358:1909-18.
 16. Luebeck EG, Moolgavkar SH. Multistage carcinogenesis and the incidence of colorectal cancer. *Proc Natl Acad Sci USA* 2002;99:15095-100.
 17. Vickers M. Estimation of the number of mutations necessary to cause chronic myeloid leukaemia from epidemiological data. *Br J Haematol* 1996;94:1-4.
 18. Frank SA. Age-specific acceleration of cancer. *Curr Biol* 2004;14:242-6.
 19. Frank SA. Age-specific incidence of inherited versus sporadic cancers: a test of the multistage theory of carcinogenesis. *Proc Natl Acad Sci USA* 2005;102:1071-5.
 20. Michor F, Iwasa Y, Nowak MA. The age incidence of chronic myeloid leukemia can be explained by a one-mutation model. *Proc Natl Acad Sci USA* 2006;103:14931-4.
 21. Vickers MA. JAK2 617V>F positive polycythemia rubra vera maintained by approximately 18 stochastic stem-cell divisions per year, explaining age of onset by a single rate-limiting mutation. *Blood* 2007;110:1675-80.
 22. Cazzaniga G, Dell'Oro MG, Mecucci C, Giarin E, Masetti R, Rossi V, et al. Nucleophosmin mutations in childhood acute myelogenous leukemia with normal karyotype. *Blood* 2005;106:1419-22.
 23. Moran P. *The statistical Processes of Evolutionary Theory*. Oxford; 1962.
 24. Abkowitz JL, Catlin SN, McCallie MT, Gutter P. Evidence that the number of hematopoietic stem cells per animal is conserved in mammals. *Blood* 2002;100:2665-7.
 25. Skarberg KO. Cellularity and cell proliferation rates in human bone marrow. IV. Studies on bone marrow cellularity and erythrokinetics in hypoproliferative anaemia. *Acta Med Scand* 1974;195:313-7.
 26. Mauer AM, Lampkin BC. Proceedings: Cellular kinetics in acute leukemia. *Proc Natl Cancer Conf* 1972;7:325-31.
 27. Schessl C, Rawat VP, Cusan M, Deshpande A, Kohl TM, Rosten PM, et al. The AML1-ETO fusion gene and the FLT3 length mutation collaborate in inducing acute leukemia in mice. *J Clin Invest* 2005;115:2159-68.
 28. Kim HG, Kojima K, Swindle CS, Cotta CV, Huo Y, Reddy V, et al. FLT3-ITD cooperates with inv(16) to promote progression to acute myeloid leukemia. *Blood* 2008;111:1567-74.
 29. Gallagher R. Duelling mutations in normal karyotype AML. *Blood* 2005;106:3681-2.
 30. Jeong EG, Lee SH, Yoo NJ. Absence of nucleophosmin 1 (NPM1) gene mutations in common solid cancers. *Apms* 2007;115:341-6.
 31. Liso A, Bogliolo A, Freschi V, Martelli MP, Pileri SA, Santodirosso M, et al. In human genome, generation of a nuclear export signal through duplication appears unique to nucleophosmin (NPM1) mutations and is restricted to AML. *Leukemia* 2008. (Epub)
 32. Shiseki M, Kitagawa Y, Wang YH, Yoshinaga K, Kondo T, Kuroiwa H, et al. Lack of nucleophosmin mutation in patients with myelodysplastic syndrome and acute myeloid leukemia with chromosome 5 abnormalities. *Leuk Lymphoma* 2007;48:2141-4.
 33. Pasqualucci L, Li S, Meloni G, Schnitger S, Gattenlohner S, Liso A, et al. NPM1-mutated acute myeloid leukaemia occurring in JAK2-V617F+ primary myelofibrosis: de-novo origin? *Leukemia* 2008. (Epub)
 34. Falini B. Any role for the nucleophosmin (NPM1) gene in myelodysplastic syndromes and acute myeloid leukemia with chromosome 5 abnormalities? *Leuk Lymphoma* 2007;48:2093-95.
 35. Albiero E, Madeo D, Bolli N, Giaretta I, Bona ED, Martelli MF, et al. Identification and functional characterization of a cytoplasmic nucleophosmin leukaemic mutant generated by a novel exon-11 NPM1 mutation. *Leukemia* 2007;21:1099-103.
 36. Bolli N, Nicoletti I, De Marco MF, Bigerna B, Pucciarini A, Mannucci R, et al. Born to be exported: COOH-terminal nuclear export signals of different strength ensure cytoplasmic accumulation of nucleophosmin leukemic mutants. *Cancer Res* 2007;67:6230-7.
 37. Falini B, Albiero E, Bolli N, De Marco MF, Madeo D, Martelli M, et al. Aberrant cytoplasmic expression of C-terminal-truncated NPM leukaemic mutant is dictated by tryptophans loss and a new NES motif. *Leukemia* 2007;21:2052-4.
 38. Falini B, Mecucci C, Saglio G, Lo Coco F, Diverio D, Brown P, et al. NPM1 mutations and cytoplasmic nucleophosmin are mutually exclusive of recurrent genetic abnormalities: a comparative analysis of 2562 patients with acute myeloid leukemia. *Haematologica* 2008;93:439-42.
 39. Chou WC, Tang JL, Lin LI, Yao M, Tsay W, Chen CY, et al. Nucleophosmin mutations in de novo acute myeloid leukemia: the age-dependent incidences and the stability during disease evolution. *Cancer Res* 2006;66:3310-6.
 40. Falini B, Martelli MP, Mecucci C, Liso A, Bolli N, Bigerna B, et al. Cytoplasmic mutated nucleophosmin is stable in primary leukemic cells and in a xenotransplant model of NPMc+ acute myeloid leukemia in SCID mice. *Haematologica* 2008;93:775-9.
 41. Gorello P, Cazzaniga G, Alberti F, Dell'Oro MG, Gottardi E, Specchia G, et al. Quantitative assessment of minimal residual disease in acute myeloid leukemia carrying nucleophosmin (NPM1) gene mutations. *Leukemia* 2006;20:1103-8.
 42. Chou WC, Tang JL, Wu SJ, Tsay W, Yao M, Huang SY, et al. Clinical implications of minimal residual disease monitoring by quantitative polymerase chain reaction in acute myeloid leukemia patients bearing nucleophosmin (NPM1) mutations. *Leukemia* 2007;21:998-1004.
 43. Gilliland DG, Jordan CT, Felix CA. The molecular basis of leukemia. *Hematology (Am Soc Hematol Educ Program)* 2004;80-97.
 44. Cheng K, Grisendi S, Clohessy JG, Majid S, Bernardi R, Sportoletti P, et al. The leukemia-associated cytoplasmic nucleophosmin mutant is an oncogene with paradoxical functions: Arf inactivation and induction of cellular senescence. *Oncogene* 2007;26:7391-400.
 45. Grisendi S, Bernardi R, Rossi M, Cheng K, Khandker L, Manova K, et al. Role of nucleophosmin in embryonic development and tumorigenesis. *Nature* 2005;437:147-53.
 46. Sportoletti P, Grisendi S, Majid SM, Cheng K, Clohessy JG, Viale A, et al. Npm1 is a haploinsufficient suppressor of myeloid and lymphoid malignancies in the mouse. *Blood* 2008;111:3859-62.
 47. Grisendi S, Mecucci C, Falini B, Pandolfi PP. Nucleophosmin and cancer. *Nat Rev Cancer* 2006;6:493-505.