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Lower frequency of NPM1 and FLT3-ITD mutations in a South African adult *de novo* AML cohort

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Abstract

Introduction—Acute myeloid leukemia (AML) is a heterogeneous clonal disorder of haemopoietic progenitor cells diagnosed in individuals of any age, but with a median age of 67 years at presentation in adults. Assessment of the mutation status of Nucleophosmin protein-1 (NPM1) and FMS-like tyrosine kinase 3 internal tandem duplication (FLT3-ITD) are essential for the diagnosis, prognosis and treatment of AML.

Methods—A total of 160 *de novo* AML cases, both cytogenetically normal and abnormal, were analyzed for the presence of NPM1 and FLT3-ITD mutations and the results assessed in conjunction with epidemiological, clinical and laboratory findings.

Results—NPM1 mutations were found in 7.5%, while FLT3-ITD was present in 12% of these cases. Both of these were lower than expected. The median age at diagnosis of AML was 41 years and for the FLT3-ITD only cases, median age was 33 years; these ages were younger than expected.

Conclusion—The lower reported frequencies and younger median age at diagnosis of AML and these specific mutations may be contributed to by a number of factors including; effects of race on age of presentation, inclusion of patients diagnosed with *de novo* AML only and a generally younger median age of the South African population.

Keywords

Acute myeloid leukemia; NPM1; FLT3-ITD; South Africa; frequency

Introduction

Acute myeloid leukemia (AML) is a heterogeneous, clonal disorder which causes significant morbidity and a 5 year overall relative survival of less than 24% [1]. Cytogenetically normal

(CN) AML accounts for 40-50% of all AML cases [2, 3], in which Nucleophosmin protein-1 (NPM1) and FMS-like tyrosine kinase 3 internal tandem duplication (FLT3-ITD) gene mutations are the most common with NPM1 mutations occurring in 27-35% and FLT3-ITD in 20-40% of all AML cases [4-6]. NPM1 is considered to be one of the founder mutations along with others such as; isocitrate dehydrogenase 1 (IDH1), Deoxyribose nucleic acid (DNA) (cytosine-5)-methyltransferase 3 alpha (DNMT3A) and tet methylcytosine dioxygenase 2 (TET2), while a number of other, secondary cooperating mutations may contribute to tumor progression and patient prognosis. NPM1 and FLT3-ITD mutations have a significant clinical impact [4, 7-9] and are often reported simultaneously. The presence of a FLT3-ITD mutation is known to be a poor prognostic feature, while the presence of a FLT3-ITD mutation in combination with an NPM1 mutation negates the good prognosis and better survival reported with an NPM1 mutation alone [10, 11]. As the list of biomarkers in AML continues to expand, attempts to classify these mutations in a hierarchical manner have been made by means of a multigene analysis approach [11]. Next generation sequencing, one of the newer methods of mutation detection, may in future better define the role that each mutation plays in leukaemogenesis as well as being expected to define novel mutations [12].

Cytogenetic analysis is the gold standard for the diagnosis of specific structural mutations, complex karyotypes commonly seen in AML and by definition, determining if the case is cytogenetically normal. Up to 15% of patients with NPM1 mutations have other confirmed genetic aberrations while approximately 21% of those found to be FLT3-ITD positive will also have an abnormal karyotype [13, 14]. This group, with aberrant cytogenetics in conjunction with NPM1 and FLT3-ITD mutations, and the group where a diagnostic cytogenetic result is not available due to a laboratories failure to karyotype will require NPM1 and FLT3-ITD mutations assessment at diagnosis and not only once normal cytogenetics has been confirmed.

The effect of patient race has been demonstrated to influence the clinical outcome of several neoplasm's, such as childhood acute lymphoblastic leukemia (ALL) where Caucasian children have a more favorable prognosis than their Black counterparts despite equal standards of care [15]. In AML, the prevalence of a subtype may differ in some populations, with Caucasians being more likely to have a normal karyotype while Black patients are more likely to carry a t(15;17) or t(8;21) translocation [16]. Assessment of karyotype and the frequency of NPM1 and FLT3-ITD mutations have not recently been determined in a predominantly Black African population.

To characterize NPM1 and FLT3-ITD mutations in South Africa we analyzed a cohort of 160 adult patients newly diagnosed with AML. This sample cohort was inclusive of all cases regardless of cytogenetic findings as the aim of this assay was to assess for these mutations at diagnosis before other results were available.

Methods

Samples were collected from September 2004 to December 2009 from adult patients newly diagnosed with AML at the National Health Laboratory Services (NHLS) flow cytometry

unit at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) and these were verified to have no prior history of a clonal myeloid disorder on the laboratory information system (LIS). The study was conducted with ethics clearance from the University of the Witwatersrand Human Research Ethics Committee (clearance number M090734 under M090688).

All patients had either bone marrow aspirate (BMA) or peripheral blood samples collected at diagnosis. Negative controls were obtained from peripheral blood or a BMA sample with normal features on morphology, immunophenotyping and cytogenetic analysis. A total of 160 patient samples were included, together with 22 control samples.

The presence of FLT3-ITD and NPM1 mutations were assessed as previously described [5, 13]. Primers for FLT3-ITD span approximately 330bp and include the common internal duplication site. The primer sets for NPM1 span approximately 170bp and provided adequate cover for mutations from A-F [17]. The amplification reaction was conducted using a thermal cycler with: initial denaturing for 10min at 95°C, followed by 45 cycles at 95°C for 20s, 60°C for 60s and 72°C for 60s and a final cycle of 30min at 72°C. The polymerase chain reaction (PCR) master mix consisted of 25mmol/l (2.0 µl) of MgCl₂, 2.5mmol/l (0.75 µl) dNTP, 1.35 µl of 10xPCR buffer, 11.9µl of sterile water, 20pmol (0.5 µl) of NPM1 Forward and 20pmol (0.5 µl) of NPM1 Reverse primers or 20pmol (0.5 µl) of FLT3 Forward and 20pmol (0.5 µl) of FLT3 Reverse primers and Amplitaq Gold 0.5 µl (2.5U). A total of 200ng of genomic DNA from the sample material made up to 2.5 µl was then added to this master mix.

The labeled PCR product samples were diluted to 1:30 with distilled water. Of these dilutions, 1µl was mixed with 8.5µl of HiDi formamide and 0.5µl of Genescan ROX 350 internal size standards and loaded in 96 well plates (Applied Biosystems, Foster City, CA). The prepared samples were then heated to 95°C for 2 minutes and then snap chilled on ice for 5 minutes directly afterwards. These samples were then separated by means of the capillary electrophoresis based system on the 3130 Genetic Analyzer using the POP7™ conformation analysis polymer (Applied Biosystems, Foster City, CA) and the fragment analysis default method as per the manufacturer's instructions. The raw data was then analyzed using the GeneMapper version 2.0 software (Applied Biosystems, Foster City, CA).

Cytogenetic analysis by G-banding was performed on BMA samples using standard methods and according to the Association for Clinical Cytogenetics (ACC) standard, with a minimum of 20 metaphases examined with at least 10 fully analyzed in new acute leukemia samples. Specimens with less than 10 metaphases for analysis were excluded [18]. Those with confirmed abnormal Fluorescence In Situ Hybridisation (FISH) analysis results were included in the cytogenetically abnormal group even though not all of these had a karyotype result available.

Patient demographics and initial laboratory parameters at diagnosis were compared according to the mutation status of NPM1 and FLT3-ITD. Categorical variables were compared using the Fischer's exact test and continuous variables using the Kruskal-Wallis

test and the Mann-Whitney U test using GraphPad Prism version 4.00 for Windows software (GraphPad Software, San Diego, CA). Differences between the results of comparative tests were considered statistically significant if the 2-sided *p* value was less than 0.05. In consultation with the Data Management and Statistical Analysis Unit of the University of the Witwatersrand, a hot-deck imputation method was used to allocate race to cases with missing information [19]. This method correlates cancer cases with missing race values against a reference database containing surnames with known race. Surnames absent from the reference database are retained in the group 'race unknown'. Preference was given to the recorded race on the laboratory records.

The date of diagnosis was defined as the date of hospital admission when the diagnosis of AML was first made. In South Africa, records of patients who are lost to follow up (LTFU) is limited and in this context a proxy for the date of LTFU was defined as the date of the last investigation registered on the NHLS LIS. The data was obtained from the NHLS corporate data warehouse (CDW) which allowed for a data search using various patient identifiers across the Gauteng provincial hospital laboratories. In addition all the 160 patients had their laboratory records assessed by means of a manual search of the laboratory system. The results of the different search methods were compared to confirm accuracy.

Results

A total of 160 cases defined as newly diagnosed AML were assessed. The results were reported as; mutation positive for both (NPM1-mut/FLT3-ITD), mutation positive for either FLT3-ITD (FLT3-ITD only) or NPM1 (NPM1-mut only) and wild type (wt) for both (NPM1-wt/FLT3-wt). This revealed a frequency of 12% (19/160) for FLT3-ITD only and 7.5% (12/160) for NPM1-mut only (Figure 1). FLT3-ITD positivity was present in 43% (9/21) of those that were NPM1 mutation positive. The median age at diagnosis for AML was found to be 41 years (range: 17-81). Those who were mutation positive for both (NPM1-mut/FLT3-ITD) had a significantly older median age of 63 years (range: 38-67) compared to those who were FLT3-ITD only and those who had neither mutation (NPM1-wt/FLT3-wt). Those who were FLT3-ITD only had a median age of 33 years (range: 19-66) and NPM1-mut positive only cases had a median age of 40 years (range: 21-75) (Figure 2). This cohort of 160 cases had 48% (77/159) male patients and 52% (82/159) females of which 114 were Black (B), 18 were White (W) or Caucasian, 13 were Colored (C) and 12 were Asian (A), and 3 could not be classified based on surname alone (Table 1). A significant difference was noted amongst the different race groups when comparing the mutation categories of NPM1-mut/FLT3-ITD vs. NPM1-wt/FLT3-wt. Further analysis indicated that NPM1-wt/FLT3-wt category had a higher ratio of Black and Colored patients as compared to the NPM1-mut/FLT3-ITD category.

FBC results at diagnosis were assessed with white cell count (WCC) results available for 97% (155/160) of cases and hemoglobin (Hb) and platelet count results available for 96% (154/160) cases. The median WCC was $12.3 \times 10^9/l$ (0.69-582), the median Hb was 7.6 g/dl (range: 2.5-16) and the median platelet count was $32 \times 10^9/l$ (range: 3-1149). A blast count was available for 99% (159/160) of cases with a median of 58% (range: 7-97) at diagnosis.

Analysis of the FBC based on the presence of NPM1 and FLT3-ITD revealed no significant difference between any of the 4 mutation categories (Table 2).

Of the NPM1-mut positive cases, only 19% (4/21) had a confirmed normal karyotype, while 67% (14/21) had no cytogenetics, FISH or real-time quantitative (RQ-PCR) results available. Cytogenetics results were considered absent if less than 10 metaphases were available for analysis as a result of poor growth or the request was omitted at presentation. Of the FLT3-ITD cases, 36% (10/28) had cytogenetic abnormalities detected while 11% (3/28) were CN and 54% (15/28) had either failed cytogenetic results no genetic analysis requested at all (Table 2).

Of the 130 AML patients with LTFU data available 40% (52/130) were LTFU 40 days after their diagnostic admission date. By day 500, 80% (104/130) of the patients were LTFU, with only 5% (6/130) of patients having been followed up for longer than 3 years. In addition, of these 130 patients, only 8% (10/130) were still confirmed to be alive in the last year (Figure 3).

Discussion

An overall assessment of the frequency and general patient characteristics including mutation profiles of *de novo* AML in a South African cohort has not been reported. FLT3-ITD occurred significantly more often ($p<0.003$) when a NPM1 mutation was identified. NPM1 mutations are the primary events that precede the acquisition of FLT3-ITD explaining the increased frequency of FLT3-ITD in NPM1 mutation positive cases [22]. The frequency of the FLT3-ITD and NPM1 mutations in this South African cohort was found to be 12% (19/160) and 7.5% (12/160) respectively, while 5.6% (9/160) of cases had dual positivity (NPM1-mut/FLT3-ITD). A lower frequency for FLT3-ITD positivity has been previously reported in cohorts from Asia compared to European populations; suggesting that differences in race may contribute to AML pathogenesis as well as mutation frequency [13, 23-25]. AML diagnosed in the African-American population is less often cytogenetically normal and by association less likely to have high frequencies of those mutations commonly seen in this intermediate risk category, including NPM1 and FLT3-ITD mutations [16]. As the cohort in this study consisted of predominantly Black Africans, race may be contributing to the decreased frequencies of the NPM1 and FLT3-ITD mutations, thus confirming these previously described findings [16]. In addition, the NPM1 mutation increases in frequency as the populations age increases [13, 26]; this could explain the lower frequency of NPM1-mut in this cohort with a considerably younger median age at diagnosis. FLT3-ITD frequency did not increase as the cohorts age increased, similar to that reported previously [5].

The median age of diagnosis of AML in this cohort was young at 41 years. A younger median age of diagnosis is recorded in adult AML cohorts that include only *de novo* AML cases compared to those which are inclusive of secondary AML cases [27, 28]. There have been reports of AML being diagnosed at a significantly younger age in African Americans, in some of these instances assessment of the general population also revealed a younger age of the population as a whole [16, 29, 30]. The black African predominance in this cohort

may explain the age disparity as compared to other non-African studies. In addition, the median age of the general South African population is 22.5 years and is younger than that seen in European countries [31-33]. Furthermore, FLT3-ITD only cases were younger than expected with a median age of 33 years [4], such a trend in this sub-category has been described previously [25]. An increase in the number of older patients with both mutations occurring simultaneously was observed, with those ≥ 60 years accounting for 53% (8/15). This was found to be statistically significant when compared to those with FLT3-ITD mutations only ($p < 0.0005$) and those with neither NPM1 nor FLT3-ITD mutations ($p < 0.02$).

Mutated NPM1 has been observed more frequently in females, in patients presenting at higher median age, with leucocytosis, thrombocytosis and higher bone marrow blast counts [4]. FLT3-ITDs are commonly associated with a leucocytosis and an increased blast percentage in both the peripheral blood and bone marrow [25, 34]. These findings were present in this cohort.

Noted in this study is the limitation of small sample size, which is especially evident when these cases are analyzed in subgroups. Another limitation of the study was the number of adult AML patients with available karyotypes at diagnosis. The presence of FLT3-ITD, NPM1 gene mutation status and abnormal cytogenetics interact in such a manner that the determination of the presence or absence of all three of these parameters is important and dependent on each other for patient outcome assessment. A number of the biomarkers recently identified also require incorporation into this list of important parameters to be used for better management for these patients [11]. Going forward a larger prospective trial with a more inclusive panel of mutations is expected to provide more comprehensive insights into the status of AML in South Africa.

Although survival data was not directly available for part of the cohort, the LTFU data was a novel strategy to provide an indication of the likelihood of survival in this cohort. Decreased survival has been reported in patients of African and Hispanic descent with acute leukemia, despite adjusting for individual and genetic factors; this detail should be assessed for in the South African population [35]. The follow up of AML patients is less than ideal, with a number of factors influencing this. Many of the patients LTFU on the LIS, in the first 40 days, are likely to either demise in hospital during the first admission post the AML diagnosis, or alternatively might be down referred for palliative care.

Some of the reasons for this initial high mortality include a lack of adequate supportive care, limited isolation facilities and barrier nursing, the presence of concomitant diseases, delay in diagnosis and initiation of therapy in patients requiring referral from primary or secondary medical facilities to tertiary centres. A delay in accessing health care in developing countries is common, with up to 95% of cancer patients being diagnosed late with a consequent poor outcome [36]. During this delay nearly half of these patients would have sought advice from a traditional healer or private medical general practitioner [37]. Once patients have received initial induction therapy, many do not return for consolidation. This may be influenced by social factors including the cost of transport, distance to hematology-oncology centres, the need to return to work and the presence or absence of an adequate home support structure. In addition, the response to therapy and prognosis may differ due to differences in drug

metabolism enzyme systems, gene polymorphisms, epigenetic differences and frequency of certain gene mutations which affect prognosis [38]. Also, many patients that do achieve remission after induction can only be offered standard chemotherapy regimens as further management, rather than individualized therapy targeting their specific mutation profile or if indicated allogeneic stem cell transplantation.

It is well known that the diagnosis, assessment of severity and prognostication of most diseases requires not only multiple special investigations but a detailed history and clinical examination. This is especially true for a complicated disease such as AML, easily evidenced by the WHO classification where numerous factors are incorporated into diagnostic algorithms to make the final diagnosis [6]. Not all individuals will be classified into a definitive category and indeed, whole groups of patients may differ from the norm. In particular, this needs to be taken into account when assessing minority groups which are often not included in the studies used to establish international guidelines. Currently there is limited data available describing AML in South Africa. This study not only provides information about specific gene mutations but also gives insight into the epidemiology and patient characteristics of those adults diagnosed with *de novo* AML.

Conclusion

The diagnosis of *de novo* AML in South Africa is made at a young age with lower than expected frequencies of NPM1 and FLT3-ITD possibly affected by race and other factors such as the younger median age of the South African population. AML in South Africa does not have a good prognosis and this is especially evident in those groups in which molecular results predict a good outcome. Although, the value of molecular testing cannot be disputed, there appears to be a number of social factors which limits the quality of care which is provided. As the diagnostic needs are beginning to be met by laboratories the limitations on the provision of appropriate therapeutic strategies as well methods to allow close follow up of patients should also be addressed.

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RCM designed the research study, performed the research, analysed the data and wrote the paper; TMW and SCC designed the research study, assisted with the research and analysed the data; MB assisted with research and analysed the data; AT and VK assisted with performing the research. SN assisted in obtaining data. All co-authors contributed to manuscript revision and have approved the final version.

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Figure 1.

Schematic representation of the distribution of AML patients by mutation sub-category. The patient cohort of 160 *de novo* AML cases revealed the presence of FLT3-ITD only in 12% of cases and the presence of NPM1-mut only in 7.5% of the cohort. FLT3-ITD positivity was present in 43% of those that were NPM1 mutation positive

Figure 2.

Distribution of each mutation category according to age. Those patients with either a single mutation or neither mutation have a peak increase in AML at 20-29 years. Those with neither mutation have a second peak of AML diagnosed at 40-49 years while those with both mutations present concurrently are more likely to be diagnosed with AML later in life with a peak seen at 70-79 years of age

Figure 3.

Time to lost to follow up (LTFU) from time of diagnosis (n=130/160). Data source, laboratory information system corporate data warehouse search. A total of 40% of all patients were LTFU by day 40 and 50% of patients were LTFU by day 87. By day 500, 80% of the patients were LTFU, only 5% were followed up for more than 3 years and 8% were confirmed to be alive within the last year

Table 1

Distribution of mutation categories according to gender, age and race

Number (n)	AML n=160	NPM1-mut/FLT3-ITD n=9	NPM1-wt/FLT3-ITD n=19	NPM1-mut/FLT3-wt n=12	NPM1-wt/FLT3-wt n=120	p-value
Gender [*]						
Male/Female (ratio)	77/82 (0.93:1)	4/5 (0.5:1)	7/12 (0.6:1)	33/67 (0.49:1)	62/57 (1.0:92)	<0.33
Age ^{*†}						
Median	41	63	33	39.5	41	<0.0005
Range	17-81	38-67	19-66	21-75	17-81	<0.0019
Race [‡]						
B/W/C/A/U	114/18/13/12/3	3/1/4/1/0	16/2/0/1/0	9/1/0/1/1	86/14/9/9/2	<0.02

n = number; B = Black; W = White; C = Coloured; A = Asian; U= Unknown. The colored population in South Africa refers to a group of South Africans whose origins are mostly from Europe, Khoisan and Bantu tribes and are therefore considered to be of mixed ancestry [20]. The majority of Asians in South Africa are of Indian descent [21, 31].

^{*} There is missing data for gender and age for a single patient of the total cohort; this patient forms part of the NPM1-wt/FLT3-wt category

[†] Age assessment revealed significant differences between the different categories when comparing mutation category NPM1-mut/FLT3-ITD vs. NPM1-wt/FLT3-ITD and when comparing mutation category NPM1-mut/FLT3-ITD vs. NPM1-wt/FLT3-wt.

[‡] The different race groups also revealed a significant difference between the NPM1-mut/FLT3-ITD vs. NPM1-wt/FLT3-wt mutation groups.

Table 2

Laboratory parameters including karyotype at diagnosis for each mutation category

Number (n)	AML n=160	NPM1-mut/FLT3-ITD n=9	NPM1-wt/FLT3-ITD n=19	NPM1-mut/FLT3-wt n=12	NPM1-wt/FLT3-wt n=120	p-value
Median Hb (g/dl) (range)	7.6 (2.5-16)	8.2 (5.3-10.9)	8.5 (3.0-13.6)	7.1 (3.8-10.7)	7.5 (2.5-16)	0.5
NR*	6	0	0	1	5	
Median Plt (x10 ⁹ /l) (range)	32 (3-1149)	35 (14-157)	43 (9-157)	23 (9-128)	31 (3-1149)	0.46
NR	6	0	0	1	5	
Median WCC (x10 ⁹ /l) (range)	12.3 (0.69-582)	76.6 (4.92-376)	42.5 (2.2-582)	30.8 (3.41-78.4)	11.8 (0.69-459.4)	0.06
NR	5	0	0	0	5	
Median blasts (range)	58 (7-97)	84 (23-95)	70 (20-95)	60 (9-83)	58 (7-72)	0.17
NR	1	0	0	0	5	
Normal Karyotype	17	1	2	3	11	
Abnormal Karyotype	77	1	9	2	65	0.12
No cytogenetics [†] , FISH or RQ-PCR	66	7	8	7	44	

Hb = hemoglobin; NR = no result; Plt = platelet count; WCC = white cell count

* NR no result was available for some of the patients' laboratory parameters; the number of results not available differed for each parameter. NR available is indicated below the respective parameter.

FISH= Fluorescent in situ hybridization; RQ-PCR= real time quantitative polymerase chain reaction

[†] No cytogenetics available was a result of either no cytogenetics being requested or growth failure