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ORIGINAL ARTICLE

Next-generation sequencing with a 54-gene panel identified unique mutational profile and prognostic markers in Chinese patients with myelofibrosis

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Abstract

Current prognostication in myelofibrosis (MF) is based on clinicopathological features and mutations in a limited number of driver genes. The impact of other genetic mutations remains unclear. We evaluated for mutations in a myeloid panel of 54 genes using next-generation sequencing. Multivariate Cox regression analysis was used to determine prognostic factors for overall survival (OS) and leukaemia-free survival (LFS), based on mutations of these genes and relevant clinical and haematological features. One hundred and one patients (primary MF, $N = 70$; secondary MF, $N = 31$) with a median follow-up of 49 (1–256) months were studied. For the entire cohort, inferior OS was associated with male gender ($P = 0.04$), age > 65 years ($P = 0.04$), haemoglobin < 10 g/dL ($P = 0.001$), *CUX1* mutation ($P = 0.003$) and *TP53* mutation ($P = 0.049$); and inferior LFS was associated with male gender ($P = 0.03$), haemoglobin < 10 g/dL ($P = 0.04$) and *SRSF2* mutations ($P = 0.008$). In primary MF, inferior OS was associated with male gender ($P = 0.03$), haemoglobin < 10 g/dL ($P = 0.002$), platelet count < 100×10^9/L ($P = 0.02$), *TET2* mutation ($P = 0.01$) and *CUX1* mutation ($P = 0.01$); and inferior LFS was associated with haemoglobin < 10 g/dL ($P = 0.02$), platelet count < 100×10^9/L ($P = 0.02$), *TET2* mutations ($P = 0.01$) and *CUX1* mutations ($P = 0.04$). These results showed that clinical and haematological features and genetic mutations should be considered in MF prognostication.

Keywords Myelofibrosis · Primary · Secondary · Next-generation sequencing · Prognosis

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Background

The classical *BCR-ABL1*-negative myeloproliferative neoplasms (MPN) comprise polycythaemia vera (PV), essential thrombocythaemia (ET) and primary myelofibrosis (MF). Patients with PV and ET may progress into secondary MF. Somatic gene mutations in haematopoietic stem cells (HSCs) drive clonal myeloproliferation. Different patterns of gene mutations determine the disease phenotype and clinical course. MF is clinically and biologically the most heterogeneous subtype of MPN, with the worst long-term outcome. In MF, disease progression is the major cause of morbidity and mortality. Leukaemic transformation accounts for approximately one third of deaths in primary MF, post-PV MF and post-ET MF [1–3]. The identification of factors predictive of survival and transformation into acute myeloid leukaemia (AML) is necessary to guide therapy.

Prognostic models for MF that incorporate clinical and molecular characteristics have been developed to predict survivals and leukaemic transformation. These models have largely been based on the mutation status of key driver genes including *JAK2*, *CALR* and *MPL* and a small panel of genes (referred to as high-molecular-risk (HMR) genes) including *ASXL1*, *EZH2*, *SRSF2* and *IDH1/2* [4–6]. Recent studies have identified recurrent mutations of genes involved in epigenetic regulation, histone modification, transcription and RNA splicing in various myeloid malignancies. The prognostic significance of these genetic mutations in MF has not been clearly defined.

In this study, we analysed a cohort of patients with primary and secondary MF for mutations in a comprehensive panel of genes implicated in myeloid neoplasms. The prognostic significance of gene mutations was also determined in conjunction with relevant clinical and haematological parameters.

Materials and methods

Patients Consecutive patients with MF diagnosed and followed up from January 1994 to November 2016 were analysed. Their pathological features were reviewed by two pathologists (J.C.C.S, H.W.I). For patients with primary MF, only cases of overt fibrotic phase according to the 2016 revised World Health Organization (WHO) criteria were included [7]. For post-PV MF and post-ET MF, the diagnoses were made according to the International Working Group for Myelofibrosis Research and Treatment (IWG-MRT) criteria [8]. Clinical data were independently reviewed by two investigators (H.G., H.W.I.). The International Prognostic Scoring System (IPSS), dynamic IPSS (DIPSS) and DIPSS-plus, determined at the time when marrow samples for genetic analysis were obtained, were analysed together with other clinical and haematological characteristics [1, 9, 10]. This study was approved by the institution review board of the Hong Kong West Cluster and the University of Hong Kong.

Management Patients were risk-stratified according to IPSS, DIPSS and DIPSS-plus. For patients with low or intermediate-1 scores, best supportive care was given. In patients with progressive leucocytosis requiring cytoreduction, hydroxyurea was the standard treatment. In patients with thrombocytosis not optimally treated by hydroxyurea without undue leucocyte suppression, anagrelide was used. Before 1996, melphalan and radioactive phosphorus were still used. These agents were abandoned after 1996 because of their leukaemogenic potentials. Allogeneic haematopoietic stem cell transplantation (HSCT) was recommended to patients who were eligible (age ≤ 60 years with an HLA-matched sibling or unrelated voluntary donor) if they had intermediate-2 to high-risk IPSS/DIPSS/DIPSS-plus scores.

Next-generation sequencing of a 54-gene panel Targeted next-generation sequencing (NGS) was performed on DNA samples from diagnostic bone marrow aspirates. DNA was extracted by QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and fragmented by Covaris S2 or KAPA Frag Enzyme (KAPA Biosystems, Cape Town, South Africa). Indexed DNA libraries were prepared from the fragmented DNA by KAPA Hyper Prep Kit or KAPA HyperPlus kit (KAPA Biosystems). A custom DNA target capture panel containing 54 genes was designed based on human reference hg19 assembly (Target Capture Probe Design & Ordering Tool, Integrated DNA Technologies, Coralville, IA, USA). The panel comprised *ABL1*, *ASXL1*, *ATRX*, *BCOR*, *BCORL1*, *BRAF*, *CALR*, *CBL*, *CBLB*, *CBLC*, *CDKN2A*, *CEBPA*, *CSF3R*, *CUX1*, *DNMT3A*, *ETV6*, *EZH2*, *FBXW7*, *FLT3*, *GATA1*, *GATA2*, *GNAS*, *HRAS*, *IDH1*, *IDH2*, *IKZF1*, *JAK2*, *JAK3*, *KDM6A*, *KIT*, *KMT2A*, *KRAS*, *MPL*, *MYD88*, *NOTCH1*, *NPM1*, *NRAS*, *PDGFRA*, *PHF6*, *PTEN*, *PTPN11*, *RAD21*, *RUNX1*, *SETBP1*, *SF3B1*, *SMC1A*, *SMC3*, *SRSF2*, *STAG2*, *TET2*, *TP53*, *U2AF1*, *WT1* and *ZRSR2* (Supplemental file 1). All exons of the 54 candidate genes were examined, with a total of 1959 probes covering 187.1 kb. Solution-based hybridization capture was performed on the DNA libraries at 65 °C for 4 h. The enriched libraries were sequenced in the Illumina HiSeq platform (Illumina, San Diego, CA, USA). FASTQ files containing at least 1.1 million raw reads were generated for each sample for bioinformatic analysis.

Bioinformatic analysis The sequenced reads were checked for quality by fastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and subjected to adaptor trimming and removal of low-quality bases by Trimmomatic [11]. They were aligned to the GRCh37/hg19 human reference sequence (2009) using the Burrows-Wheeler Aligner (BWA) (version 0.7.15) [2] with default parameters. Picard tools (version 2.4.1; http://broadinstitute.github.io/picard) were used to mark duplicates and perform indel realignment. Base recalibration was performed by GATK (version 3.6) tools [12]. Single nucleotide variants (SNV) and indels were called independently by VarScan2 [13] at a sensitivity of 5% on the pileup files generated by Samtools [14] and HaplotypeCaller in GATK tools. Detection of *FLT3* internal tandem duplication was performed by both Pindel [15] and GATK HaplotypeCaller. The resulting variants were annotated by ANNOVAR [16], using the databases of esp6500siv2 (http://evs.gs.washington.edu/EVS/), 1000g2014oct, clinvar_20160302, dbnsfp30a and cosmic77 [12, 17–20]. Briefly, all variants detected were processed with the variant frequency filter (< 1% in 1000 genomes), variant allele frequency filter (> 5%) and coverage filter (> 500×). The filtered variants were then annotated based on variant database searches (COSMIC, TCGA, locus-specific databases), variant prediction (variant type, Polyphen2, SIFT, LRT, Mutation Taster) and

actionability (genes or variants with prognostic and therapeutic significance) [21].

Statistical analysis All data were censored on 31 December 2016. Overall survival (OS) was defined as the time from diagnosis to death (event), HSCT (censored) or latest follow-up (censored). Leukaemia-free survival (LFS) was defined as the time from diagnosis to leukaemic transformation (event), death (event), HSCT (censored) or latest follow-up (censored). Survivals were estimated using the Kaplan-Meier method, and compared with the log-rank test and modelled by the Cox proportion hazard model. Parameters evaluated for potential impact on survivals included gender (male versus female), age (> versus ≤ 65 years), constitutional symptoms (present versus absent), haemoglobin (> versus ≤ 10 g/dL), leucocyte count (≤ versus $> 25 \times 10^9$/L), platelet count (< versus $\geq 100 \times 10^9$/L), circulating blast percentage (< versus ≥ 1%), red cell transfusion (dependence versus independence), IPSS (low/intermediate-1 versus intermediate-2/high), DIPSS (low/intermediate-1 versus intermediate-2/high), karyotype (presence versus absence of complex or ≥ one of +8, −7/7q-, i(17q), −5/5q-, 12p-, inv(3) or 11q23 rearrangement), and somatic mutations (present versus absent) of each of the 54 genes and HMR gene mutation (involvement of none versus 1 versus ≥ 2 genes). Prognostic parameters with P values of < 0.1 on univariate analysis were included in multivariate analysis by Cox proportional hazard regression. Association between categorical variables was determined with the Fisher's exact and χ^2 tests. Differences in the co-occurrence of mutations were determined with the McNemar test and the Cochran's Q test. Association between continuous variables was determined with the Mann-Whitney U or Kruskal-Wallis tests. Statistical analyses were performed using the SPSS software version 23. P values (two-tailed) of < 0.05 were considered statistically significant. Charts and heatmaps were constructed using GraphPad Prism version 7.02 and R package, respectively. Gene association and correlation was performed by GeneNet V1.2.13 (R package) utilizing graphical Gaussian model. Covariance selection was used to determine gene association. Concentration graph analysis was used to determine the gene relevance network, generating a covariance matrix for Circos plot (Circos software) [22].

Results

Clinical and haematological characteristics and outcome One hundred and one patients (primary MF, $N = 70$; post-PV MF, $N = 14$; post-ET MF, $N = 17$) were studied (Table 1). Karyotype was available in 53 patients at diagnosis (Supplemental file 2). With a median follow-up of 46 (1–256) months, there were 49 deaths. Transformation into AML occurred in 18 patients. Thromboembolic events occurred in 15 patients (arterial thrombosis, $N = 8$; venous thromboembolism, $N = 7$). Hydroxyurea was the most common drug used ($N = 58$), followed by anagrelide ($N = 9$). Melphalan and radioactive phosphorus were each used in one patient (Supplemental file 3). Allogeneic HSCT was performed in 20 patients (sibling donor, $N = 13$; voluntary unrelated donor, $N = 7$). There was no mortality, but eight patients relapsed after a median follow-up of 8.5 (3–51) months post-HSCT. The 5-year and 10-year OS were 66.3% and 35.4% (Fig. 1a). The 5-year and 10-year LFS were 63.5% and 34.9% (Fig. 2a). There was no significant difference in OS and LFS between primary and secondary MF ($P = 0.87$ and $P = 0.86$, respectively) (Figs. 1b and 2b).

Mutational characteristics Of the 54 genes tested, 39 genes showed mutations (Fig. 3a) (Supplemental file 4). Five of the 101 patients did not show mutations in any of the genes tested (Fig. 3b). The majority of cases had mutations in ≥ 1 genes (1 mutation: $N = 14$, 13.9%; 2 mutations: $N = 38$, 37.6%; 3 mutations: $N = 18$, 17.8%; 4 mutations: $N = 15$, 14.9%; 5 mutations: $N = 7$, 6.9%; ≥ 6 mutations: $N = 4$, 4%).

Functional patterns of mutations Mutated genes were grouped according to their putative cellular functions (Supplemental file 5). Genes involved in cellular signalling were most frequently mutated ($N = 91$; 90%), including *JAK2* V617F ($N = 58$; 57%), *CALR* ($N = 22$; 22%) and *MPL* ($N = 6$; 6%). These mutations were mutually exclusive (Supplemental file 5). The second most frequently mutated genes were those involved in histone modification ($N = 36$; 36%) and transcription regulation ($N = 36$, 36%). The next most frequently mutated genes were those involved in DNA methylation ($N = 23$; 23%) and RNA splicing ($N = 20$; 20%). Genes in the cohesin complex ($N = 4$; 4%) were least frequently mutated.

Concurrent mutations Several recurring concurrent mutations were observed, including *JAK2*V617F/*TET2* (16 patients, 15.8%), *JAK2*V617F/*ASXL1* ($N = 12$; 12%), *CALR*/*ASXL1* ($N = 10$; 10%) *JAK2*V617F/*CUX1* ($N = 6$; 6%), *JAK2*V617F/*EZH2* ($N = 6$; 6%), *JAK2*V617F/*RUNX1* ($N = 5$; 5%), *JAK2*V617F/*SF3B1* ($N = 5$; 5%), *JAK2*V617F/*SETBP1* ($N = 4$; 4%) and *JAK2*V617F/*ZRSR2* ($N = 4$; 4%) (Fig. 3c). In patients with *JAK2*V617F or *CALR* mutant MF, there were significant differences between the proportion of each co-occurring mutation ($P < 0.001$). The co-occurrence of *JAK2*V617F with *TET2* or *ASXL1* was not significantly different ($P = 0.48$). However, the co-occurrence of mutants was statistically significant for *JAK2*V617F with *CUX1* ($P = 0.03$), *EZH2* ($P = 0.03$), *RUNX1* ($P = 0.01$), *SF3B1* ($P = 0.03$), *SETBP1* ($P = 0.01$) and *ZRSR2* ($P = 0.004$).

Table 1 Clinical and haematological features in 101 patients with myelofibrosis

Clinical and haematological parameter	All patients ($N = 101$)	PMF ($N = 70$)	Post-PV MF ($N = 14$)	Post-ET MF ($N = 17$)	P value[#]
Gender					
Male	62	47	8	7	0.13
Female	39	23	6	10	
Median age (range) (years)	60 (26–89)	60 (26–89)	62.5 (41–86)	57 (35–86)	0.68
Age > 65 years	44	34	5	5	0.29
Presence of constitutional symptoms	69	46	11	12	0.62
Presentation blood counts					
Median haemoglobin (range) (g/dL)	10.3 (3–18.5)	10.6 (3–17)	11.0 (7–18.5)	9.7 (7.1–14.5)	0.18
Haemoglobin < 10 g/dL	46	30	5	11	0.20
Median leucocyte count (range), $\times 10^9$/L	12.1 (1.47–177.4)	12.4 (1.5–177.4)	15.8 (2.5–38.5)	9.5 (1.64–17.4)	0.07
Leucocyte count > 25×10^9/L	16	13	3	0	0.14
Circulating blast > 1%	33	20	5	8	0.33
Median platelet count (range), $\times 10^9$/L	344 (19–1720)	436 (19–1720)	328 (93–617)	314 (59–1361)	0.37
Platelet count < 100	19	15	2	2	0.59
Red cell transfusion dependence	38	28	4	6	0.71
IPSS					0.22
Low	11	5	2	4	
Intermediate-1	25	22	2	1	
Intermediate-2	23	15	4	4	
High	42	28	6	8	
DIPSS					0.58
Low	12	6	2	4	
Intermediate-1	28	23	3	2	
Intermediate-2	45	31	7	7	
High	16	10	2	4	
Unfavourable karyotype*	4	3	0	1	0.58
Median follow-up duration (range) (months)	46 (1–256)	50 (1–256)	43.5 (6–246)	44 (5–76)	0.40

IPSS International Prognostic Scoring System, *DIPSS* Dynamic International Prognostic Scoring System, *PMF* primary myelofibrosis, *PV* polycythaemia vera, *ET* essential thrombocythaemia

*Karyotype was performed in 53 patients at diagnosis

[#] P value refers to the difference between PMF, post-PV MF and post-ET MF

Prognostic factors for OS For the entire cohort, on univariate analysis, inferior OS was associated with male gender ($P = 0.01$), haemoglobin < 10 g/dL ($P = 0.001$), red cell transfusion dependence ($P = 0.04$), intermediate-2/high-risk IPSS ($P = 0.001$), intermediate-2/high-risk DIPSS ($P = 0.002$), *CUX1* mutation ($P = 0.04$), *U2AF1* mutation ($P = 0.009$), *ETV6* mutation ($P < 0.001$), *SRSF2* mutation ($P = 0.02$), *TP53* mutation ($P < 0.001$) and the presence of ≥ 2 HMR mutations ($P = 0.023$) (Supplemental file 6). On multivariate analysis, risks remaining significant included male gender ($P = 0.04$), age > 65 years ($P = 0.04$), haemoglobin < 10 g/dL ($P = 0.001$), *CUX1* mutation ($P = 0.003$) and *TP53* mutation ($P = 0.05$) (Table 2) (Fig. 1c–g). For cases of primary MF, on univariate analysis, inferior OS was associated with male gender ($P = 0.03$), haemoglobin < 10 g/dL ($P = 0.001$), platelet count < 100×10^9/L ($P = 0.004$), intermediate-2/high-risk IPSS ($P = 0.008$), intermediate-2/high-risk DIPSS ($P = 0.03$), *EZH2* mutation ($P = 0.04$), *CUX1* mutation ($P = 0.03$), *ETV6* mutation ($P = 0.005$) and *TP53* mutation ($P = 0.004$) (Supplemental file 7). On multivariate analysis, risks remaining significant were male gender ($P = 0.03$), haemoglobin < 10 g/dL ($P = 0.002$), platelet count < 100×10^9/L ($P = 0.02$), *TET2* mutation ($P = 0.01$) and *CUX1* mutation ($P = 0.01$) (Table 2) (Fig. 1h–l). For secondary MF, on univariate, inferior OS was associated with age > 65 years ($P = 0.03$), leucocyte count > 25×10^9/L ($P = 0.03$), *ASXL1* mutation ($P = 0.04$), *U2AF1* mutation ($P = 0.007$), *ETV6* mutation ($P = 0.01$) and *TP53* mutation ($P = 0.007$) (Supplemental file 8). On multivariate analysis, none of these factors were significant.

Prognostic factors for LFS For the whole cohort, on univariate analysis, inferior LFS was associated with male gender ($P = 0.02$), haemoglobin < 10 g/dL ($P = 0.002$), intermediate-2/

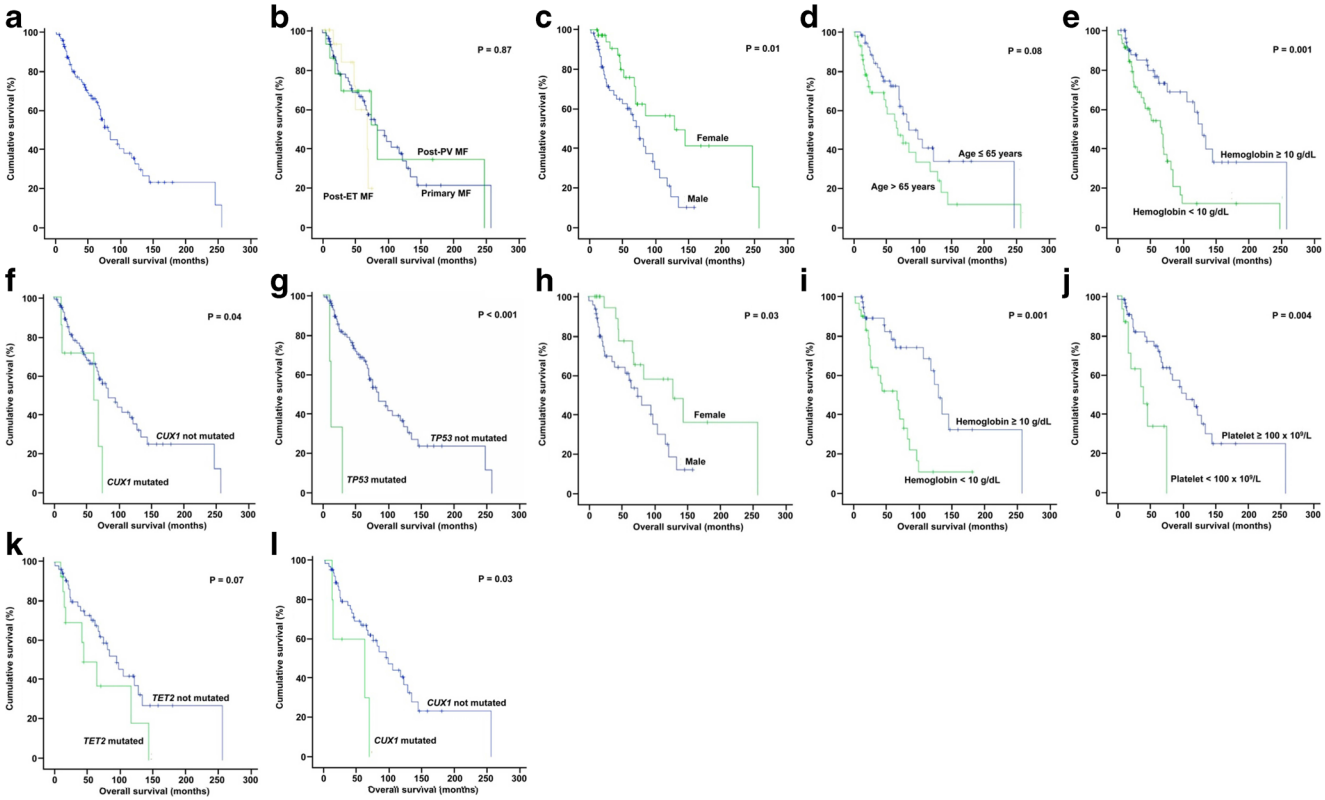

Fig. 1 Overall survivals of patients with myelofibrosis. **a** Overall survival (OS) of the entire cohort of patients with primary and secondary myelofibrosis (MF). **b** Overall survival (OS) was not significantly different between patients with primary and secondary myelofibrosis (MF). **c**–**g** For the entire cohort, multivariate analysis showed that overall survival (OS) was significantly inferior in patients with the male gender ($P = 0.04$) (**c**), age > 65 years ($P = 0.04$) (**d**), haemoglobin < 10 g/dL ($P = 0.001$) (**e**), *CUX1* mutation ($P = 0.003$) (**f**) and *TP53* mutation ($P = 0.05$) (**g**). *P* values shown in the figures were those in univariate analysis. **h**–**l** In primary myelofibrosis, multivariate analysis showed that overall survival (OS) was significantly inferior in patients with the male gender ($P = 0.03$) (**h**), haemoglobin < 10 g/dL ($P = 0.002$) (**i**), platelet count < 100 × 109/L ($P = 0.02$) (**j**), *TET2* mutation ($P = 0.01$) (**k**) and *CUX1* mutation ($P = 0.01$) (**l**). *P* values shown in the figures were those in univariate analysis

high-risk IPSS ($P = 0.001$), intermediate-2/high-risk DIPSS ($P = 0.003$), *U2AF1* mutation ($P = 0.01$), *ETV6* mutation ($P < 0.001$), *SRSF2* mutation ($P = 0.03$), *IDH2* mutation ($P = 0.03$), *TP53* mutation ($P < 0.001$) and the presence of ≥ 2 HMR mutations. On multivariate analysis, risks remaining significant included male gender ($P = 0.03$), haemoglobin < 10 g/dL ($P = 0.03$) and *SRSF2* mutation ($P = 0.008$) (Fig. 2c–e). For primary MF, on univariate analysis, inferior LFS was associated with male gender ($P = 0.04$), haemoglobin < 10 g/dL ($P = 0.001$), platelet count < 100×10^9/L ($P = 0.005$), intermediate-2 and high-risk IPSS ($P = 0.007$), intermediate-2 and high-risk DIPSS ($P = 0.03$), *EZH2* mutation ($P = 0.03$), *CUX1* mutation ($P = 0.03$), *ETV6* mutation ($P = 0.007$) and *TP53* mutation ($P = 0.004$). On multivariate analysis, risks remaining significant included haemoglobin < 10 g/dL ($P = 0.02$), platelet count < 100×10^9/L ($P = 0.02$), *TET2* mutation ($P = 0.01$) and *CUX1* mutation ($P = 0.04$) (Fig. 2f–i). For secondary MF, on univariate analysis, inferior LFS was associated with age > 65 ($P = 0.04$), leucocyte count > 25×10^9/L ($P = 0.04$), *U2AF1* mutation ($P = 0.007$), *ETV6* mutation ($P = 0.01$) and *TP53* mutation ($P = 0.007$). On multivariate analysis, none of the factors were significant.

Discussion

In this study, employing a panel comprising genes implicated in myeloid neoplasms, we identified mutations in 39 genes in our MF cohort. These genes can be segregated into functional groups with pathogenetic and potential therapeutic significance.

Mutations of genes involved in cytokine signalling were most prevalent, with the three most common genes being *JAK2*, *CALR* and *MPL*. Their frequencies of mutations in the entire cohort (*JAK2*V617F 57.4%; *CALR* 21.8%; *MPL* 5.9%) and in primary MF (*JAK2*V617F 52.9%; *CALR* 21.4%; *MPL* 7.1%) were similar to those reported previously in other patient cohorts [2, 3, 5]. Our sequencing platform also evaluated all *JAK2* coding exons. Overall, 19 patients (18.8%) (PMF, $N = 12$; post-PV MF, $N = 1$; post-ET MF, $N = 6$) had *JAK2* variants other than *JAK2*V617F. These comprised *JAK2*G127D ($N = 16$), *JAK2*K857E ($N = 1$), *JAK2*V392M ($N = 1$) and *JAK2* I724T ($N = 1$). These *JAK2* variants co-existed with *JAK2*V617F ($N = 10$) and *CALR* mutations ($N = 4$). Recently, the variants *JAK2*G571S, *JAK2*G335D, *JAK2*V625F and *JAK2*F556V were detected in 4 of 49

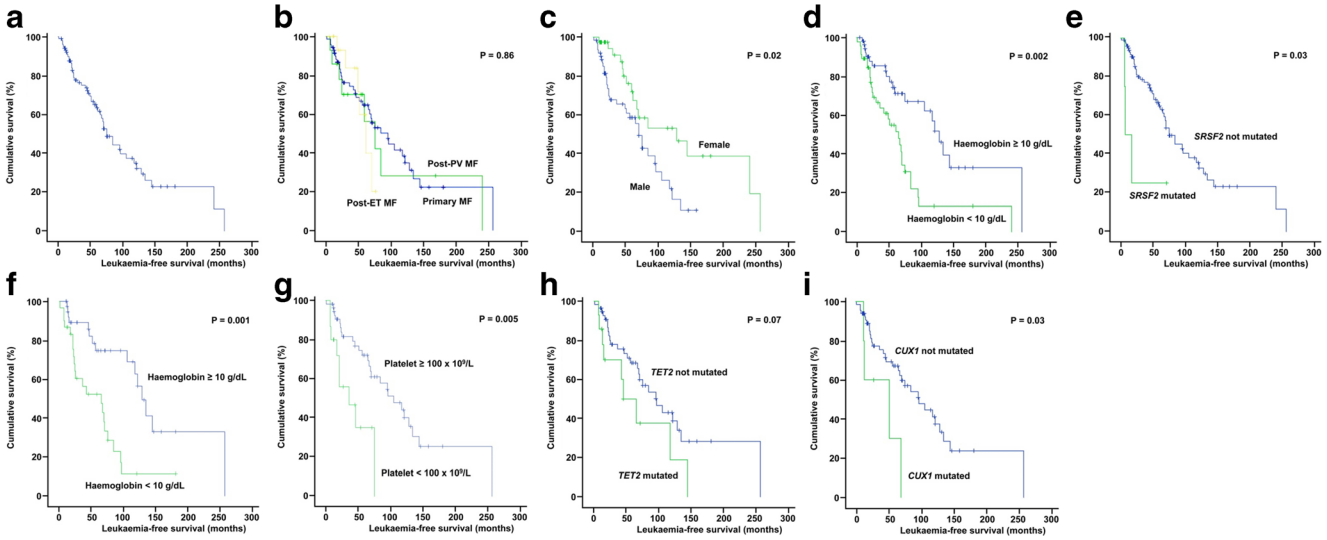

Fig. 2 Leukaemia-free survivals (LFS) of patients with myelofibrosis. **a** Leukaemia-free survival (LFS) of the entire cohort of patients with primary and secondary myelofibrosis (MF). **b** Leukaemia-free survival (LFS) was not significantly different between patients with primary and secondary myelofibrosis. **c**–**e** For the entire cohort, multivariate analysis showed that leukaemia-free survival (LFS) was significantly inferior in patients with haemoglobin < 10 g/dL ($P = 0.007$) (**c**), *IDH2* mutation ($P = 0.001$) (**d**) and *TP53* mutation ($P = 0.04$) (**e**). *P* values shown in the figures were those in univariate analysis. **f**–**h**. In primary myelofibrosis, multivariate analysis showed that leukaemia-free survival (LFS) was significantly inferior in patients with haemoglobin < 10 g/dL ($P = 0.02$) (**f**), *RUNX1* mutation ($P = 0.02$) (**g**) and *DNMT3A* mutation ($P = 0.004$) (**h**). *P* values shown in the figures were those in univariate analysis

patients with ET and PMF triple negative for *JAK2*V617F, CALR and MPL mutations [23]. The *JAK2* variants *JAK2*V625F and *JAK2*F556V activated JAK-STAT signalling and so were pathogenetically significant [23]. *JAK2*G127D detected in 16 of our cases has been reported in more than 1% in normal East Asian population and most likely represents germline polymorphism [24]. *JAK2*V392M is present in COSMIC (version 81) and is likely benign. *JAK2*I724T and *JAK2*K857E are not present in COSMIC (version 81), so that their clinical relevance is uncertain. The significance of the other *JAK2* variants detected in our cases requires further functional validation.

Mutations in genes involved in epigenetic regulations, including histone modification and DNA methylation, were the second most common. *ASXL1* mutations were observed in 31 patients (31%) (primary MF, $N = 25$; post-PV MF, $N = 2$; post-ET MF, $N = 4$). *ASXL1* encodes the chromatic-binding protein associated with polycomb repressive complex (PRC) 1 and 2,

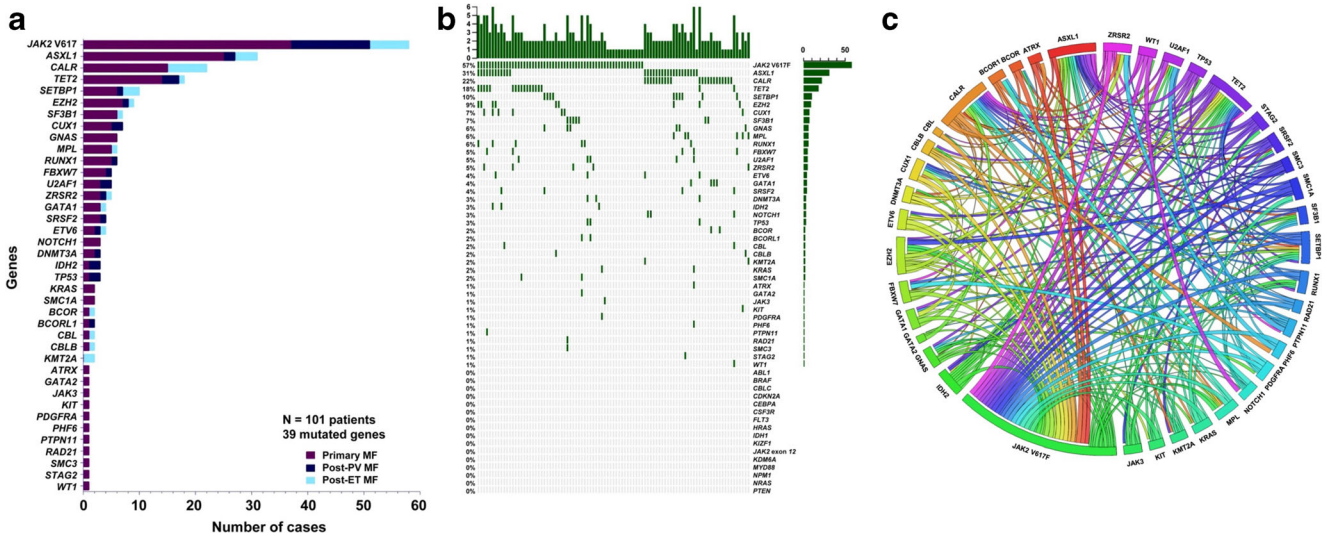

Fig. 3 Mutations in a 54-gene panel in myelofibrosis. **a** Frequency of mutations in 39 genes in 101 patients with primary myelofibrosis (PMF), post-polycythaemia vera myelofibrosis (post-PV MF) and post-essential thrombocythaemia myelofibrosis (post-ET MF). **b** Heatmap showing the frequency and distribution of mutations of 54 genes in 101 patients with myelofibrosis. Each small grid represents one individual patient. **c** Circos plot showing concurrent mutations in 101 patients with myelofibrosis. The presence of two or more concurrent mutations is shown

Table 2 Significant prognostic indicators on multivariate analysis

Prognostic indicators	Number of patients	Overall survival		Leukaemia-free survival	
		P value	HR (95% CI)	*P* value	HR (95% CI)
Entire cohort (101 patients)					
Male gender	62	0.04	2.07 (1.02–4.19)	0.03	2.08 (1.06–4.08)
Age > 65 years	44	0.04	2.06 (1.02–4.18)	–	–
Haemoglobin < 10 g/dL	46	0.001	3.81 (1.76–8.25)	0.03	2.53 (1.06–6.01)
CUX1 mutation	7	0.003	5.29 (1.78–15.71)	–	–
TP53 mutation	3	0.049	10.95 (1.01–118.36)	–	–
SRSF2 mutation	4	–	–	0.008	6.23 (1.60–24.24)
Primary myelofibrosis (70 patients)					
Male gender	47	0.03	2.52 (1.09–5.81)	–	–
Haemoglobin < 10 g/dL	30	0.002	3.5 (1.58–7.79)	0.02	3.89 (1.22–12.40)
Platelet $< 100 \times 10^9$/L	15	0.02	3.07 (1.18–7.95)	0.02	3.14 (1.20–8.18)
TET2 mutation	14	0.01	3.11 (1.30–7.47)	0.01	3.48 (1.34–9.02)
CUX1 mutation	5	0.001	8.18 (2.33–28.70)	0.04	6.48 (1.14–36.87)

HR hazard ratio, *CI* confidence interval

and is involved in leukaemia initiation and progression. Nonsense mutations/indels in *ASXL1* have been reported to occur in up to 25% of primary MF and 1–3% of PV or ET [25, 26]. *EZH2* mutations were seen in nine patients (9%) (primary MF, $N = 7$; post-PV, $N = 1$; post-ET, $N = 1$). *EZH2* encodes the histone methyltransferase of H3K27, which is associated with leukaemia initiation and progression. *EZH2* mutations have been reported to occur in 5–10% of primary MF and 3% of PV [25, 26]. DNA methylation-associated genes mutated in our cohort included *TET2*, *IDH2* and *DNMT3A*. Interestingly, *IDH1* mutations were not observed. *TET2* mutations were observed in 18 patients (18%) (primary MF, $N = 14$; post-PV MF, $N = 3$; post-ET MF, $N = 1$). *TET2* mutations are associated with disease initiation and progression, and were observed previously in 17%, 16% and 5% of primary MF, PV and ET, respectively [25, 26]. *DNMT3A* and *IDH2* mutations were each seen in three patients. *DNMT3A* mutations occurred in 5–10% of patients with MPN and were associated with disease initiation. *IDH1* and *IDH2* mutations occurred in up to 3% of patients with primary MF and were associated with disease initiation and progression [25, 26].

Mutations in genes regulating transcription were the third most common. *SETBP1* mutations were seen in ten patients (10%) (primary MF, $N = 6$; post-PV MF, $N = 1$; post-ET MF, $N = 3$). *SETBP1* mutations have been described in myelodysplastic syndrome (MDS)/MPN, chronic myelomonocytic leukaemia, atypical chronic myeloid leukaemia, juvenile myelomonocytic leukaemia and secondary AML. In leukaemia, *SETBP1* mutations are associated with adverse cytogenetics including −7/del(7q) and i(17q), and an aggressive disease course [19, 27]. In PMF, *SETBP1* mutation has been reported in 2.5% of patients [28]. Mutations in *CUX1* gene, a transcription regulator of *TP53* and *ATM* located on 7q22, were seen in seven patients (6.9%) (primary MF, $N = 5$; post-ET MF, $N = 2$) [26]. CUX1 activates phosphoinositide-3-kinase signalling, thereby playing important roles in tumourigeneis [29]. *CUX1* mutations, previously reported in < 3% of MPN, are associated with del(7q) and leukaemic transformation [26, 29, 30]. The *CUX1* mutants detected in our study have not been reported previously in myeloid malignancies. In a meta-analysis, *CUX1* mutations, mostly missense mutations, were found in 1–5% of various malignancies [29]. Significantly inferior survivals were seen in 2519 patients with MDS, MDS/MPN and AML harbouring *CUX1* truncating mutations [29]. *RUNX1* mutations were seen in six patients (6%) (primary MF, $N = 5$; post-PV MF, $N = 1$). Mutations in *RUNX1*, a master transcription regulator of haematopoiesis, were observed in < 3% of MPN, and were associated with leukaemic transformation [26, 31].

Genes involved in RNA splicing mutated in our cohort included *SF3B1*, *U2AF1*, *ZRSR2* and *SRSF2* [25, 26]. Mutations in the cohesion complex genes *SMC1A*, *RAD21*, *SMC3* and *STAG2* were only observed in five patients with primary MF. Their significance in MF is unknown. The putative significance of other less frequently mutated gene remains undefined (Supplemental file 9) [26, 32–41].

We evaluated by multivariate analysis the impact of genetic mutations on survivals together with other clinical and

haematological parameters [1, 9, 10]. While conventional clinical and haematological parameters remained important in prognostication (entire cohort: male gender, age > 65 years, haemoglobin < 10 g/dL and platelet count < 100×10^9/L for inferior OS; male gender and haemoglobin < 10 g/dL for inferior LFS) (primary MF: male gender, haemoglobin < 10 g/dL, platelet < 100×10^9/L for inferior OS; haemoglobin < 10 g/dL and platelet < 100×10^9/L for inferior LFS), gene mutations emerged as important risk factors for unfavourable outcome. Male gender was shown to adversely impact on OS and LFS in the entire cohort and also in patients with primary MF. Male gender has not been shown in previous studies of myelofibrosis to be prognostically relevant. The biological basis for this observation remains to be determined. Previous studies have shown that primary MF patients triple negative for *JAK2*V617F, *CALR* and *MPL* mutations have the worst outcome [5, 42, 43]. A recent study evaluating 685 patients with secondary MF showed that non-mutated *CALR* impacted negatively on OS [2]. Furthermore, in another study of post-ET MF patients, *JAK2*V617F alone, or triple negativity for *JAK2*V617F, *CALR* and *MPL* mutations, had significantly worse outcome than *CALR* mutated cases [44]. In our cohort, triple negativity for *JAK2*V617F, *CALR* and *MPL* mutations was not an adverse prognostic factor. This might be explained by the fact that a significant proportion of *JAK2*V617F, *CALR* or *MPL* mutant patients had concurrent mutations in transcription factors and epigenetic regulators that were associated with aggressive disease course, rendering their prognoses similar to that of triple-negative patients. The negative prognostic impact of ≥ 2 HMR mutations had been shown in large cohorts of patients with primary MF [4, 6, 45]. In more recent prognostic models incorporating clinical parameters and gene mutations in PMF, the absence of CALR type 1 mutation and the presence of one or more HMR mutations (*ASXL1*, *EZH2*, *SRSF2*, *IDH1/2*) were independent adverse prognostic indicators [46, 47]. In another prognostic model solely dependent on genetic factors, a very high risk (VHR) karyotype, the absence of type 1/type 1-like *CALR* mutations and the presence of *ASXL1*, *SRSF2* or *U2A*F1Q157 mutations were independent adverse prognostic indicators regardless of clinical features [47, 48]. In post-PV and post-ET MF, the prognostic impact of HMR mutations had also been validated [49]. The presence of HMR mutations had variable impact in our cohort of patients. The adverse prognostic impact of *SRSF2* and ≥ 2 HMR mutations was seen in our entire cohort of MF patients on univariate but not multivariate analysis. *SRSF2*, *IDH2* and ≥ 2 HMR mutations were associated with worse LFS on univariate analysis, with *SRSF2* remaining significant for inferior LFS on multivariate analysis. In patients with primary MF, *EZH2* mutation was associated with worse OS and LFS on univariate but not multivariate analysis.

There are unique adverse molecular prognostic markers identified in this study. *CUX1* mutation was associated with inferior OS in the whole cohort and primary MF patients, as well as inferior LFS in primary MF patients. Previous studies had shown *CUX1* mutations to be of negative prognostic significance in MDS and AML [29], and we showed for the first time that *CUX1* mutations were associated with inferior survivals in MF. The variants reported in this study are not frequently noted in Western populations. Because samples tested were archival, we were unable to obtain paired normal samples to confirm the somatic nature of these variants. This is particularly so in patients with *CUX1* mutations, who had all died. It has been shown that CUX1 is a key tumour suppressor gene and inactivating mutations of *CUX1* are frequently noted in myeloid malignancies [50]. CUX1 knockdown also leads to an MDS/MPN phenotype in mice [50]. Hence, the significance of *CUX1* variants in our patients with MF requires further functional validation. *TET2* mutations resulted in inferior OS and LFS in primary MF patients. The adverse prognostic impact of *TET2* mutations in MPN had not been consistently shown by previous studies [51, 52]. *TP53* mutations were associated with inferior OS in all MF patients, consistent with the association of *TP53* mutations with poor-risk cytogenetics and increased risk of leukaemic transformation [53].

This study has some limitations. The number of patients investigated in this study was relatively small, and might have impacted on the correlation of genetic mutations with outcome. Furthermore, we have not directly verified that the mutants observed were not rare germline variations using non-myeloid tissues, although variants in transcription factors and tumour suppressor genes in MF are rare. Hence, our observations will need to be validated in future studies.

In conclusion, we have characterized the genomic alterations and prognostic factors in a large cohort of Chinese patients with MF. Using a sensitive NGS platform examining all exons of a broad panel of genes, we have uncovered mutations of prognostic relevance not previously described in MF. This was exemplified by the unique prognostic relevance of *CUX1*, *RUNX1*, *TET2*, *DNMT3A* and *U2AF1* mutations. Our findings call for further studies validating the frequencies, biological significance and prognostic impacts of these mutations. Prospective studies are needed to determine if MF patients with these gene mutations ought to be managed differently, in order to mitigate their potentially negative prognostic implications.

Author contributions Harinder Gill: treated the patients, analysed the data, wrote and approved the manuscript.

Ho-Wan Ip: performed the experiments, wrote and approved the manuscript.

Rita Yim: performed the experiments, wrote and approved the manuscript.

Wing-Fai Tang: performed the experiments and approved the manuscript.

Herbert H. Pang: performed the experiments and approved the manuscript.

Paul Lee: performed the experiments and approved the manuscript.

Garret M.K. Leung: treated the patients and approved the manuscript.
Jamilla Li: treated the patients and approved the manuscript.
Karen Tang: treated the patients and approved the manuscript.
Jason C.C. So: performed the experiments and approved the manuscript.
Rock Y.Y Leung: performed the experiments and approved the manuscript.
Jun Li: performed the experiments and approved the manuscript.
Gianni Panagioutou: performed the experiments and approved the manuscript.
Clarence C.K. Lam: performed histopathological analysis and approved the manuscript.
Yok-Lam Kwong: treated the patients, wrote and approved the manuscript.

Compliance with ethical standards Patients gave informed consent to treatment. This study was approved by the institution review board of the Hong Kong West Cluster and the University of Hong Kong, and was conducted according to the Declaration of Helsinki.

Conflict of interest The authors declare that they have no conflict of interest.

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