Pathophysiologic and clinical implications of molecular profiles resultant from deletion 5q

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Summary

Background Haploinsufficiency (HI) resulting from deletion of the long arm of chromosome 5 [del(5q)] and the accompanied loss of heterozygosity are likely key pathogenic factors in del(5q) myeloid neoplasia (MN) although the consequences of del(5q) have not been yet clarified.

Methods Here, we explored mutations, gene expression and clinical phenotypes of 388 del(5q) vs. 841 diploid cases with MN [82% myelodysplastic syndromes (MDS)].

Findings Del(5q) resulted as founder (better prognosis) or secondary hit (preceded by TP53 mutations). Using Bayesian prediction analyses on 57 HI marker genes we established the minimal del(5q) gene signature that distinguishes del(5q) from diploid cases. Clusters of diploid cases mimicking the del(5q) signature support the overall importance of del(5q) genes in the pathogenesis of MDS in general. Sub-clusters within del(5q) patients pointed towards the inherent intrapatient heterogeneity of HI genes.

Interpretation The underlying clonal expansion drive results from a balance between the “HI-driver” genes (e.g., CSNK1A1, CTNNA1, TCERG3) and the proapoptotic “HI-anti-drivers” (e.g., RPS14, PURA, SIL1). The residual essential clonal expansion drive allows for selection of accelerator mutations such as TP53 (denominating poor) and CSNK1A1 mutations (with a better prognosis) which overcome pro-apoptotic genes (e.g., p21, BAD, BAX), resulting in a clonal expansion. In summary, we describe the complete picture of del(5q) MN identifying the crucial genes, gene clusters and clonal hierarchy dictating the clinical course of del(5q) patients.

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Introduction
Deletions in the long arm of chromosome 5 [del(5q)] can be found in different myeloid neoplasia (MN) and are the most common karyotypic alterations in myelodysplastic syndromes (MDS). Del(5q) can be found alone or with other cytogenetic abnormalities and is associated with specific morphologic features. However, del(5q) is the only cytogenetic abnormality used to define a phenotypically distinct WHO MDS subtype, originally described by Van den Berghe and colleagues in 1974. Del(5q) encompasses two commonly deleted regions (CDRs). The centromeric CDR on 5q31.2-5q31.3 (CDR-1) is associated with a higher-risk MDS or acute myeloid leukaemia (AML). The distal CDR spanning 1.5Mb on 5q32-3q32.2 (CDR-2) is present in the classical 5q- Syndrome and conveys better prognosis. The clinical del(5q) phenotype may also be affected by the actual boundaries of the deletion, along with other chromosomal defects and somatic mutations co-existing in a hierarchical relationship with del (5q). Significant progress in the understanding of the disease mechanisms resulting from del(5q) has been made. However, it has not been entirely clarified which haploinsufficient (HI) genes on 5q are associated with the disease development and malignant progression. Here, we examine a large cohort of del(5q) patients to clarify the molecular relationships of these lesions with other somatic defects and determine their impact on the resultant expression patterns and the morphologic and clinical phenotypes.

Methods
Patients and samples: A total of 400 samples (388 patients and 12 follow-up samples) with MNs (myeloid neoplasms) with del(5q) (Table 1) determined by conventional G-banding cytogenetics and fluorescence in situ hybridization (FISH) were included in the study. A cohort of patients with MN diploid status for 5q [n = 844 samples (840 patients and four follow-up samples)] were also collected for comparison purposes. All samples included were profiled for gene sequencing and cytogenetics was available for all.

Ethics Information: All samples were collected with previous written informed consent in accordance with institutional ethics committee approvals following the revised Declaration of Helsinki. A total of 51 samples were collected at the Josep Carreras Leukemia Research Institute (2010/4102/I) in between 2009 and 2014 on behalf of the MDS Spanish group, 185 samples were collected at the Cleveland Clinic (IRB-5024) in between 2002 and 2017, and 1008 samples were collected at the MLL Munich Leukemia laboratory in between 2005 and 2018. We think our cohort is indeed representative because: (i) cohorts from 3 centres of excellence were collected in the same time period and all the diagnostic criteria and data collected were synchronized; (ii) our sample size is large enough to accommodate for the biologic diversity, including rare subtypes and; (iii) is not hampered by a single institutional bias.

Next-Generation Sequencing (NGS): Samples underwent targeted deep sequencing (tumour sample; n = 141), whole exome sequencing (WES; tumour and germline sample; n = 95) or whole genome sequencing (WGS; tumour sample; n = 1008). To combine the data from the three platforms (Table 5) we selected 181 genes (on the basis of their implication in the

Research in context

Evidence before this study
The prevailing theory in del(5q) is that haploinsufficiency (HI) stemming from deletion and not simply loss of heterozygosity (LOH) is the culprit in clonal evolution. To date no haploinsufficient gene has been found to be the pivotal leukaemogenic factor conveying growth advantage, but various other genes have been found to be important for phenotypic features or for propensity to acquire subsequent specific lesions. This study represents the most comprehensive compendium of genomic and RNA sequencing data of patients with MDS carrying del(5q). Unlike previous studies, we based our analysis on an innovative copy number adjustment that mitigates the clonality effect on the haploinsufficient genes on 5q.

Added value of this study
Our study based on a large cohort, has defined distinct expression signatures of molecular subtypes of del(5q) according to the position in the clonal hierarchy and correcting previous assumptions that del(5q) was the ultimate ancestral lesion at the level of a stem cell. We have also provided a minimal haploinsufficient (HI) expression signature of del(5q) including most indicative genes and their pathophysiologic role. Furthermore, we defined HI-driver (clonal expansion) and HI-anti-drivers (brakes, proapoptotic) genes and their complex interactions in del(5q) neoplasia affecting the clinical course. Clonal drive overcomes proapoptotic pressure (HI-anti-drivers) through escape mechanisms promoted by accelerator events (e.g., CSNK1A1, TP53 mutations).

Implications of all the available evidence
Our analysis of a compendium of del(5q) genomics confirms some of the previous findings, revises previous assumptions, discovers new haploinsufficient candidate genes and precisely circumscribes molecular relationships (e.g., del(5q) with TP53 mutations) and generates a minimalistic expression signature of del(5q).
pathogenesis of MN; Table S2). After variant calling, all samples were analysed together based on a strict in-house variant filtering process in line with current scientific standards. There was no institutional bias in terms of distribution of somatic mutations. RNA-sequencing (RNA-seq) was performed only on samples collected at the MLL Munich Leukemia laboratory.

**Clonal hierarchy:** To determine dominant/ancestral and sub-clonal/secondary hits and del(5q) for each patient: Copy Number Variation from WES data was calculated based on the allelic imbalance of informative heterozygous SNPs within the 5q deleted region in WES germ line samples. Copy Number Variation analysis from WGS data: Copy number variant (CNV) calls were created using GATK4 (version 4.0.2.1), following the “best practices” somatic CNV workflow (https://software.broadinstitute.org/gatk/best-practices/).

RNA-Seq: Total RNA from whole bone marrow (BM) samples was extracted. RNA-Seq libraries were constructed from ribosomal RNA depleted RNA using TruSeq Total Stranded RNA kit (Illumina) and sequenced on a NovaSeq 6000 platform.

### Haploinsufficiency expression analysis: RNA-Seq results from 995 patients (170 del(5q) and 825 diploid patients) with MN were analysed. Patients were further classified according to the karyotype, BM blast % and TP53 mutational status. After data processing (supplemental methods), a total of 148 del(5q) and 752 diploid patients were included in the analysis. Gene expression was measured in counts per million (CPM), assigned to each gene (see supplemental methods).

### Adjustments for del(5q) clonality: Linear models were fit per gene using the R package limma with log2(CPM+1) as the response variable and del(5q) CNV clonality (centred at 50% clonality), del(5q) status (isolated, +1, CK), diploid karyotype (normal, 1+, 2+, CK), TP53 mutational status (wild type, mutant) and blast risk [BM blasts %: low (<5%), high (>5%)] as covariates.

### Del(5q) haploinsufficient signature: Sparse Prediction Modeling: models for predicting del(5q) vs. diploid status were built using a Bayesian sparse logistic regression approach as implemented in the R package HTLR.

### Table 1: Clinical features of del(5q) cohort.

|                  | del(5q) cohort (n = 388) | del(5q) + 1 | del(5q) in CK |
|------------------|--------------------------|-------------|--------------|
| n (%)            | 188 (49)                 | 36 (9)      | 164 (42)     |
| Female, n (%)    | 132 (70)                 | 23 (64)     | 71 (43)      |
| Age, median years (min-max) | 73 (35 – 94)          | 73 (42 – 88) | 73 (24 – 89) |

| Classification, n (%) |
|-----------------------|
| Low Risk              |
| MDS low risk          | 154 (82)                  | 25 (69) | 37 (22) |
| CMML-1                | 1 (0.5)                   | 1 (3)   | 0       |
| High risk             |
| MDS high risk         | 20 (11)                   | 5 (14)  | 78 (48) |
| CMML-2                | 0                         | 0       | 0       |
| sAML                  | 10 (5)                    | 4 (11)  | 30 (18) |
| pAML                  | 3 (1.5)                   | 1 (3)   | 11 (7)  |
| MDS/MPN-U             | 0                         | 0       | 8 (5%)  |

| Blood counts (%)     |
|----------------------|
| Anaemia (<10g/dL)    | 117 (69)                  | 19 (58) | 106 (68) |
| Thrombocytopenia (<100 x 10^9/L) | 17 (10)        | 12 (36) | 113 (72) |
| Thrombocytosis (>450 x 10^9/L)   | 24 (14)                 | 2 (6)   | 2 (1)   |
| Neutropenia (<1.8 x 10^9/L)  | 51 (46)                  | 17 (55) | 88 (72) |

| % BM blasts, median (min-max) |
|-------------------------------|
| 3 (0 – 76)                    | 3 (0 – 45)               | 8 (0 – 87) |

CK: Complex Karyotype: ≥ 5 cytogenetic alterations; Low risk: MDS low-risk: MDS-SLD (MDS with single lineage dysplasia), MDS-RS (MDS with ring sideroblasts), MDS-MLD (MDS with multilineage dysplasia), MDS del(5q) (MDS with isolated del(5q)), MDS-U (unclassifiable MDS); CMML-1 (chronic myelomonocytic leukemia-1); High risk: MDS high-risk: MDS-EB-1 (MDS with excess blast-1), MDS-EB-2 (MDS with excess blast-2); CMML-2 (chronic myelomonocytic leukemia-2); sAML (secondary AML); pAML (primary AML); MDS/MPN-U (myelodysplastic/myeloproliferative neoplasm unclassifiable). BM: bone marrow.

(CBC data available for: iso-del(5q): anemia: n = 169; thrombocytopenia: n = 169; thrombocytosis n = 169; neutropenia: n = 111; del(5q) +1: anemia: n = 33; thrombocytopenia: n = 33; thrombocytosis n = 33; neutropenia: n = 31; del(5q) in CK: anemia: n = 156; thrombocytopenia: n = 156; thrombocytosis n = 156; neutropenia: n = 122).
Figure 1. Cytogenetic and molecular characterization of 5q: (a) CNV analysis based on the cytogenetic break points was used to determine the most commonly deleted region in del(5q).
(b) Mutation distribution in isolated del(5q), compound del(5q), and diploid patients.
(c) Percentage of mutant patients in the most common mutated genes (mutations on chromosome 5 are not included).
Figure 2. Clonal architecture and subclonal hierarchy: reconstruction of the clonal hierarchy using an allelic imbalance method for WES samples (with available paired sample) and by CNV analyses for WGS, were compared to the VAF of most common mutations adjusted by zygosity and copy number.

(a) Distribution of ancestral (navy blue), co-dominant (yellow) or subclonal (light purple) del(5q) in isolated del(5q) and compound del(5q).

(b) Kaplan-Meier curves for ancestral, secondary and co-dominant del(5q) event with significant differences (Log rank test).

(c) Mutational distribution of the most representative mutations in dominant, co-dominant or subclonal del(5q) in patients with isolated del(5q) or compound del(5q).

(d) Exemplary cases of clonal architecture of isolated del(5q) patients (left) and in compound del(5q) patients (right).

(e) Percentage of clonal burden of del(5q) (red), increased subclonal mutations (yellow), and decreased subclonal mutations (blue). Lines connect paired samples. Dotted lines indicate undetectable mutation after treatment.
except for initial states, which were chosen using the bias-corrected Bayesian classification approach option. Final gene predictors were those having non-zero model coefficients (calculated using the HTLR nzero_idx function). Models were trained using MH1(50%) and MH1 (25%) data with 900 samples (148 del(5q) samples) and expression from 57 del(5q) genes. Class predictions were made using the posterior median of the regression coefficients. Prediction error was assessed using ten-fold cross validation and feature selection stability was assessed by counting the number of times the feature was selected across the ten fits.

Statistical analyses: Statistical analyses were performed on GraphPad Prism (version 8.0.2) and R statistical software packages (version 3.6.0). Chi-square or Fisher’s exact test were performed for categorical variables, while Mann-Whitney and Wilcoxon tests were used for pairwise continuous variables. The overall survival (OS) for diagnosis samples was defined from diagnosis to death or last follow-up. All deaths, whether or not related to MDS, were considered as the endpoint of the follow-up interval. Kaplan-Meier method was used to estimate the OS curves, and log rank test was used for comparisons between groups. All p-values reported to estimate the OS curves, and log rank test was used for pairwise continuous variables. The overall survival (OS) for diagnosis samples was defined from diagnosis to death or last follow-up. All deaths, whether or not related to MDS, were considered as the endpoint of the follow-up interval. Kaplan-Meier method was used to estimate the OS curves, and log rank test was used for comparisons between groups. All p-values reported in this study were two-sided and considered statistically significant when \( P < 0.05 \).

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Results

Clinical and genetic features of del(5q) patients. We included 388 patients with del(5q)-associated (Table 1) and 844 MN without del(5q) (Table S1; Figure 1a-b). mRNA-seq results from BM (obtained retrograde flushing of discarded de-identified BM harvest kits) of 64 healthy individuals [median age 59 (range, 26–85)] were included as reference. Patients were grouped according to del(5q) status: isolated del(5q) (iso-del5q) and compound del(5q) (comp-del5q); including del(5q) plus any other alteration (+1-del5q) or in the context of complex karyotype (CK-del5q) (Figure S1a-b). To delineate the boundaries of the del(5q), we conducted copy number variant (CNV) analyses using conventional cytogenetics. The CDRs in our del(5q) cohort (CDR-A; Figure 1a) included CDR-1 and CDR-2. The commonly retained regions (CRR) centromeric (p12-q13) and telomeric (q35.1-q35.3) were mostly co-deleted in CK-del5q (36% and 52% of patients, respectively; Figure 1a). NGS analysis for 181 selected genes (Table S2) yielded 2,251 somatic mutations in 1,034 (83%) cases (Table S3). While a total of 71% of patients with iso-del5q carried one mutation (12% on 5q), 8% of patients with comp-del5q harboured one mutation (8% on 5q; \( P = 0.001; \) Fisher’s exact test) but were less mutated than patients without 5q (\( P = 0.001; \) Fisher’s exact test) (Figures 1b, S1c).

For the mutational profile, we selected the most commonly mutated genes in MNS (\( n = 181 \)). Comp-del5q showed a higher frequency of TP53 mutations vs. iso-del5q and mutant diploid cases (\( P < 0.0001; \) Chi²-test), and a significantly lower frequency of hits in SF3B1, TET2, and ASXL1, vs. iso-del5q (Figure 1c). When we focused on mutations on 5q, CSNK1A1 (532) was the most commonly mutated gene in iso-del5q cases. Canonical CSNK1A1E98 (\( n = 13 \)) and CSNK1A1P440V (\( n = 2 \)) were identified. Neither canonical DDX41 (535.3) frame-shift germ line, nor somatic missense ATP-binding domain mutations were found within deleted areas and the only three DDX41 mutations in del(5q) were all heterozygous. Neither concurrent mutations showed diverse dynamics; (i) as a dominant event, more frequent in iso-del5q (61%) than in comp-del5q (46%; \( P = 0.03; \) Fisher’s exact test); (ii) as a sub-clonal event with similar frequencies in comp-del5q (27%) and iso-del5q (24%); or (iii) as a co-dominant event with unresolved rank of somatic alterations/del(5q), more commonly in comp-del5q (27%) than in iso-del5q (15%; \( P = 0.04; \) Figure 2a). Patients with a dominant del(5q) had better overall survival (OS; \( P = 0.0296; \) Kaplan-Meier, log rank test) than those with del(5q) in a co-dominant or subclonal configuration (Figure 2b). Del(5q) occurring in a dominant configuration coincided with secondary CSNK1A1 mutations (ten%) in iso-del5q, and TP53 (26%) in comp-del5q. However, when del(5q) was a sub-clonal event, it was often preceded by dominant ASXL1 mutations (26%) in iso-del5q and dominant TP53 (56%) in comp-del5q (Figure 3c). Figure 2d shows exemplary cases with ancestral iso-del5q and sub-clonal CSNK1A1 and TP53B1. When a clear clonal hierarchy could not be mathematically resolved, del(5q) co-ranked with TP53, ASXL1, and TP53B1 mutations in iso-del5q and TP53 and DNMT3A in comp-del5q.

Sequential BM and granulocyte fraction samples were available from 7 and 3 patients, respectively. Nine patients received at least 6 cycles of LEN (LEN cycles: pt1:8, pt2: 9; pt3: 6, pt4:8; pt5: 9, pt6: 13; pt7:10; pt8:7; pt9:32), and pt10 was during disease progression. Most consistently, del(5q) decreased in response to LEN (pt1, pt2, pt3, pt4, pt6, pt7). Concurrent mutations showed diverse dynamics; (i) decrease/ disappearance of mutations (e.g., DNMT3A,
Figure 3. Haploinsufficiency (HI) analysis of selected genes on 5q. (a) Box plot showing the definition of HI expression defined when the expression in del(5q) was lower than the percentile 25th of the diploid expression level.

(b) Gene expression (Log2 CPM) in del(5q) cases (pink) and diploid cases (blue). Dashed lines show median values.

(c) Exemplary cases of different correlation types between expression and clonality in isolated del(5q) and compound del(5q). Green error bar shows the expression of the given gene in diploid cases. Black error bar shows the expected expression at 50% and 100% clonality of del(5q).

(d) Heatmap using the spares model-based clustering of del(5q) and diploid patients (see methods), including the 57 HI genes. Karyotype group, TP53 mutational status, BM Blast % group and % of clonality are also depicted.
expression (Figure 3a-b). We noticed a variable land-del(5q) when it was 
/C20 genes) and 128 were deleted across all patients on 5q, 188 were located within CDR-A (CDR-1: 41, CDR-
pt3, pt4); (ii) Other genes were excluded because they were not 
clonal burden or was too variable to be pathognomonic. 
genes either did not correlate with the del(5q) 
EGR1 ploidy slope in isolated del(5q) (Figure S3, Table S4). (q23.3-q33.1) showing evidence of HI and negative 
(TAF7, EGR1) (Figure 3c). To identify causative genes, 
affected by opposing effects (DDX46, PURA), while 
(PPP2AC, RPS14), while those with a steeper slope are 
tial compensation or deletion of a methylated allele 
expected expression, due to the del(5q), may show a par-
corresponding 5q ploidy. Genes with a higher-than-
expected expression, due to the del(5q), may show a par-
HI in del(5q) patients. By comparison diploid cluster-
haploinsufficient signature of del(5q) and 
haploinsufficient patterns within del(5q). Using the 57 
HI marker genes, we performed a sparse Bayesian pre-
diction analyses to identify the minimal del(5q) gene 
signature that could distinguish del(5q) from diploid 
cases. Using the 50% clonality adjusted data, the mini-
gene signature contained UBE2D2, C5orf24, PURA, 
SIL1, and HSPA4 genes and misclassified only one del (5q) sample (0-1% observed error rate for clustering 
within 5q deleted samples; Figures 4a; S4a). Even when this five-gene signature was applied to the 25% clonality 
adjusted data, the error rate was only 6.7%. When we 
conducted pairwise associations between the 57 genes, 
we found a striking correlation with distinct groups of 
highly correlated 5q genes. Even though diploid patients 
also exhibited some significant correlations, those corre-
lations were stronger and more significant in del(5q). 
Repeating the signature search using the 25% clonality 
adjusted data resulted in a seven HI gene signature 
(PURA, SIL1, HSPA4, CSNK1A1, TCOF1, CTNNA1, and 
FAM13B) with only 11 del(5q) and eight diploid patients 
misclassified (one% error rate; Figure S4b-c). When we 
plied the model-based sparse sub-clustering within del (5q) patients a nine gene signature was found (TGFBI, 
CTNNA1, PURA, CXXC5, DCTN4, SMAD5, HARS, 
TMEM173, and RPS14) to obtain six different expression 
clusters demonstrating heterogeneity of HI within del 
(5q) patients (Figure 4b; d, Tables S7 and 8). Cluster 
comparison showed that cluster-A and cluster-E had the 
majority of CK but cluster-A showed low mutational 
burden and lowest expression of TGFBI, CTNNA1, 
and PURA. Cluster-E showed more 5q as secondary hit, 
high number of TP33 mutations (n = 20), and HI for 
SMAD5, RPS14, and TMEM173 but less profound HI of 
PURA and CXXC5. Cluster-B and cluster-F included the 
majority of isolated del(5q). Ancestral 5q hits and deeply 
depressed expression of TGFBI, CTNNA1, and PURA 
(but not CXXC5 or RPS14) were grouped in cluster-B. 
Cluster-C pattern was the most common (n = 62), with 
50% of ancestral del(5q), frequent TP33 mutations 
(n = 23) and low expression of CTNNA1, PURA, and 
HARS. Interestingly, RPS14 mRNA was not uniformly 
HI in del(5q) patients. By comparison diploid cluster-
Figure 4. Minimal gene signature of del(5q).
(a) Heat map including the del(5q) 5 gene-minimal signature according to a sparse Bayesian prediction analysis that differentiates between del(5q) and diploid patients. Expression of del(5q) patients was adjusted to a 50% del(5q) clonality representing the expression of del(5q) and diploid patients. Blue colours represent a low expression while red colours represent up-regulation of the expression (white means neutral).
(b) Heat map including the del(5q) 9 gene-minimal signature within del(5q) patients. Blue colours represent a low expression while red colours represent up-regulation of the expression (white means neutral).
two contained patients with a down-modulation of RPS14 but no relationship with p21 levels or TP53 mutations was seen in diploid cases vs. those with del(5q) and low RPS14 expression.

Pathogenic interactions. We identified several subclasses according to their function with regard to clonal advantage: (i) directly or indirectly acting proapoptotic genes (BAD, BAK1, BAX, among others) (Figure 5a) whose HI facilitates subclonal escape via e.g., hypomorphic TP53 mutations or via CSNK1A1 mutations. HI levels of CSNK1A1 may promote apoptosis (of MDM2 dephosphorylation leading to p21 increase). UBE2D2 HI in del(5q) may result in selective pressure for del(5q) CSNK1A1, CTNNA1, C5orf24 (TECRG1) genes.

We then focused on selected interactions between well-established mutations and the newly defined HI 5q genes. CSNK1A1 mutations were only found in del(5q) patients (12% vs. none in diploid cases); CSNK1A1 and TP53 mutations did not coincide; only 15% were co-mutated with TP53 (two-sided *p* value; Figure 5b). All del(5q) showed CSNK1A1 HI expression (15% of diploid patients were CSNK1A1 low expressors; *le;* ≤75th percentile; Figure 5c). Diploid CSNK1A1-le also had low expression of RPS14 (*P = 0.125; [Mann-Whitney test]; Figure 5d). Moreover, CSNK1A1 expression did not correlate with TP53 mutations (Figure 5e). Irrespective of CSNK1A1 mutations or wild type expression level, CDKN1A (p21) as a downstream effector of TP53 was generally up regulated in del(5q) except for TP53 mutants showing p21 mRNA levels comparable to those of diploid patients (*P = 0.125; [Mann-Whitney test]; Figure 5f). Similarly, del(5q) cases with HI RPS14 expression also showed decreased p21 expression (*P < 0.0001; [Mann-Whitney test]; Figure 5g).

While expression of CDC25C, DIAPH1, and RAD50 genes did not correlate with ploidy (FDR for ploidy slope (SL): 0.988; 0.072; 0.307; respectively; Figure 5h), EGR1 expression showed a positive ploidy slope (FDR SL: 0.211), but those with a relatively lower expression were 75% of TP53 mutants (40/53; *P < 0.0001; [Chi²-test]); (Figure 5i). APC, when involved in the deleted region, was indeed HI (*P < 0.0001) and correlated with the presence of CSNK1A1 mutants (*n = eight/nine*; Figure 5j). When deleted (21/167) DDX41 were indeed HI; all these patients had a CK and 18/21 of these patients also harboured TP53 mutations (Figure 5k).

**Discussion**

Del(5q) results in various biologic features due to the HI of individual genes contained in the affected region. We investigated the molecular mechanisms of del(5q) including HI genes and mutations affecting cellular function to elucidate the phenotype-genotype associations leading to apoptosis/proliferation/differentiation and disassociation in MDS.

Using an integrative-molecular approach, we demonstrated: (i) del(5q) patients have similar but less complex mutational landscapes as other MDS subtypes with mutational signatures dominated by exclusively hemizygous CSNK1A1 mutations, along with an increased frequency of TP53 mutations; (ii) a subset of HI genes (*n = 75*) was selected on significant expression reduction with a negative correlation with 5q clonality. Minimal expression signature points towards the presence of marker genes for del(5q) irrespective of their functional importance. For analytic purposes, minimal signature genes may help to design RNA based tests for del(5q) using minimal number of target genes and to identify del(5q) cells in single cell RNA-seq. While reproducibility of minimal signature may be an issue, our study has identified 77 consistently HI genes, which can be added to improve the precision of identification of del(5q); (iii) del(5q) neoplasms result from complex interaction between HI-driver (clonal expansion) and HI-anti-drivers (brakes, proapoptotic genes; (iv) clonal drive eventually overcomes proapoptotic pressure (HI-anti-drivers) through escape mechanisms promoted by accelerator events; (v) using gene clustering according to expression reduction, we identified subsets of diploid patients with 5q gene repression patterns similar to del(5q) patients; (vi) we determined a minimal HI gene signature of del(5q) involving essential genes. Integration of these findings led to a new and more complete concept of pathogenesis of del(5q).

In contrast to some previous studies postulating that del(5q) is an obligatory primary hit, we demonstrated that del(5q) is not always an initiating event. It may be preceded by other mutations (e.g., mutations in TP53), a success characterized by an early advanced phenotype. Subclonal TP53 mutations likely represent a later escape mechanism resulting from apoptotic pressure produced by HI of various genes. When del(5q) was dominant, the most common secondary hits in isolated del(5q) were mutations in CSNK1A1 [8,15] and for compound del(5q) TP53 mutations. The individual fate of the clinical course of del(5q) is thus influenced by the hierarchy and the succession of molecular events, adding further complexity to del(5q) sub-entity.

For the purpose of this discussion, we focused on the ancestral del(5q) characterizing the classical del(5q) entity (iso-del(5q)). Among HI genes involved in ancestral del(5q) there must be some HI-drivers which provide the impetus for clonal expansion, thus enabling selection needed to eventually overcome HI-anti-driver genes producing the phenotypic dysplasia and apoptosis of del(5q). Such a pressure leads to acquisition of accelerat events such as CSNK1A1 mutations, TP53 mutations or deletion, and/or monosomy 7.

Putative HI-drivers include the previously “established” del(5q) genes: CSNK1A1, CTNNA1, and...
Figure 5. Pathogenic mutational interactions in del(5q): (a) Expression of selected pro-apoptotic genes showing a significant higher expression in del(5q) compared to diploid patients.

(b) Frequency of mutant CSNK1A1 that are co-mutated with TP53 (left) and frequency of TP53 mutants that include CSNK1A1 mutations (right).

(c) Expression (Log2 CPM) of CSNK1A1 in del(5q) patients and diploid patients.

(d) RPS14 expression (Log2 CPM) comparison between diploid patients with low expression (le) of CSNK1A1 and diploid patients with normal expression of CSNK1A1.

(e) CSNK1A1 expression comparison between del(5q) patients WT (solid colour) or MT (grey stripes) for CSNK1A1, TP53. Diploid WT patient expression is also depicted.

(f) CDKN1A expression comparison between del(5q) patients WT (solid colour) or MT (grey stripes) for the CSNK1A1 and TP53 genes. Diploid WT patient expression is also depicted.

(g) CDKN1A expression in del(5q) vs. del(5q) low RPS14 expressors and diploid low RPS14 expressors (cluster-2).

(h) Correlation between the percentage of del(5q) (according to CNV analysis) and expression (Log2 CPM) of excluded genes located on 5q. Green error bar represents the expression of the given gene in diploid patients, TP53 wild type, and <5% bone marrow blasts. Black error bar represents the estimated expression of the given gene at 50% and 100% of del(5q) clonality. [Isolated del (5q) patients (blue line), compound del(5q) (pink line)].

(i) EGR1 expression in del(5q) and diploid cases TP53 mutant (solid colour) or wild type (grey stripes).

(j) Correlation between % of del(5q) (according to CNV analysis) and expression (log2 CPM). Dots represents expression and clonality of patients without deletion involving APC or DDX41.

(k) APC expression in del(5q) and diploid cases. On the right frequency of CSNK1A1 mutants and wild type in del(5q) APC low expressors.

(l) DDX41 expression in patients with del(5q) and DDX41 deleted, del(5q), and diploid cases.
also some new genes such as C3orf25 (TCERG1).20 Thus, del(5q) results in a complex interaction between the HI-anti-drivers which resist the expansion promoted by the HI-driver genes. The acquisition of accelerator hits will shift the balance between the proapoptotic and pro-proliferative functions in favour to the last. Of note is that we included as accelerators CSNK1A1 gain of function mutations which shift the balance towards pro-proliferative function Wnt/β-catenin.16 TP53 alterations may cooperate with the pro-tumorigenic effects or cytogenetic alterations like monosomy 7 or del(17p) may ultimately lead to a more aggressive phenotype. Finally, EGR1 upregulation in response to apoptotic stress will be attenuated via TP53 inactivation,21,22 consistent with our results.

HI-anti-driver genes include e.g., RPS14, HSPA4, SIL1, and UBE2D2 and all promoted the increased apoptosis in del(5q).23 Whereas we show that RPS14 is not uniformly HI, our results are otherwise consistent with earlier reports,4,32 as del(5q) cases with RPS14 deletion did harbour most of the TP53 mutations. Together with or in the absence of RPS14, HI of HSPA4 and SIL1 genes may also contribute to cell cycle arrest and apoptotic pressure in del(5q).26 PURA is another del(5q) gene which represses TP53 and BAX and thus its HI promotes apoptosis.37 Interestingly, PURA can either function as a homodimer (activating the transcription) or as a repressive heterodimer with PURB located on chromosome 7p. Simultaneous deletion of PURA and PURB correlates with an enhanced progression to sAML in MDS.34 Another HI-anti driver gene, UBE2D2 may also be consequential to del(5q) pathogenesis. Knock-down of UBE2D2 decreases TP53 ubiquitination and degradation.35 Finally, increased expression of CDKN1A (p21), caspase-3, -8, and -9 and BAX genes supports notions of high apoptotic pressure in del(5q) cells.

The recently published single cell barcoding strategy by knockout of HI loci of Csnk1a1, Apc, and Egr1 showed that only HI of Csnk1a1 resulted in a clonal expansion as shown in serial transplantations, while HI of Apc or Egr1 did not.39 Our study is compatible with these experiments also assigning a prominent role of CSNK1A1 in the clonal expansion, despite its duality in function (apoptosis vs. enhancing the Wnt/β-catenin pathway). The latter pathway can be augmented by the CSNK1A1 gain of function mutations. A similar effect may be a result of APC down-modulation in deleted cases, as previously described.40

Despite considerable effort, our study may suffer from the obvious shortcomings inherent to retrospective study with samples collected at different times. Another limitation is that genomic and expression data were based on bulk DNA and RNA. Further studies with single cell technologies are needed to determine the differences during haematopoiesis of del(5q) and non-del(5q) cells.

In summary, we provide an integrated analysis of the genome of del(5q) patients. We identified key HI genes (drivers vs. anti-drivers) and gene clusters, that together with acquired mutational events and their hierarchy might determine the clinical course and complexity of del(5q). We also provide a substrate for future generation of mouse models for confirmatory and genetics studies to further characterize the HI-drivers and HI-anti-drivers in del(5q).

**Contributors**

V.A. carried out the experimental/analytic procedures, interpreted the data, and wrote the manuscript. L.P., M. M., and V.V. collected data and performed DNA molecular studies. W.W., M.M., S.H., T.L., T.R., and J.O.B. conducted bioinformatic analysis. J.O.B. established statistical analysis and conducted haploinsufficiency analysis. V.V., L.A., B.X., A.P., J.B., M.S., provided clinical specimens and important insights on the manuscript. T.H., C.H., and W.K. provided molecular data RNA-Seq and WGS and helped to interpret results and edit the manuscript. J.P.M., T.H., and FS supervised the study and provided patient specimens and data. V.V. and J.P.M. have verified the underlying data. J.P.M. formulated and edited the manuscript. J.P.M. and F.S. equally contributed. All authors read and approved the final manuscript.

**Data sharing statement**

All information are provided in the appendix. Requests for additional information can be sent to the corresponding author. RNA-sequencing results have been reported in Hershberger CE et al., Leukemia 2021.

**Declaration of interests**

Dr. Sekeres received consultation fees from BMS/Celgene and Kurome. All the other authors declare no conflicts interests.

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Supplementary materials

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