Trisomy 12 chronic lymphocytic leukemia expresses a unique set of activated and targetable pathways

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ABSTRACT

Although trisomy 12 (+12) chronic lymphocytic leukemia (CLL) comprises about 20% of cases, relatively little is known about its pathophysiology. These cases often demonstrate atypical morphological and immunophenotypic features, high proliferative rates, unmutated immunoglobulin heavy chain variable region genes, and a high frequency of NOTCH1 mutation. Patients with +12 CLL have an intermediate prognosis, and show higher incidences of thrombocytopenia, Richter transformation, and other secondary cancers. Despite these important differences, relatively few transcriptional profiling studies have focused on identifying dysregulated pathways that characterize +12 CLL, and most have used a hierarchical cytogenetic classification in which cases with more than one recurrent abnormality are categorized according to the abnormality with the poorest prognosis. In this study, we sought to identify protein-coding genes whose expression contributes to the unique pathophysiology of +12 CLL. To exclude the likely confounding effects of multiple cytogenetic abnormalities on gene expression, our +12 patient cohort had +12 as the sole abnormality. We profiled samples obtained from 147 treatment-naïve patients. We compared cases with +12 as the only cytogenetic abnormality to cases with only del(13q), del(11q), or diploid cytogenetics using independent discovery (n=97) and validation (n=50) sets. We demonstrate that CLL cases with +12 as the sole abnormality express a unique set of activated pathways compared to other cytogenetic subtypes. Among these pathways, we identify the NFAT signaling pathway and the immune checkpoint molecule, NT5E (CD73), which may represent new therapeutic targets.

Introduction

Chromosomal abnormalities, predominantly gains and losses, are strong predictors of disease progression and survival in chronic lymphocytic leukemia (CLL). Fluorescence in situ hybridization (FISH) assays on interphase nuclei have demonstrated that approximately 80% of cases contain non-random gains or losses of chromosomal material, many with prognostic significance.1 Deletions in 13q14 (del(13q)) are most common, followed by deletions in 11q22.3-q23.1 (del(11q)), trisomy 12 (+12), and deletions in 6q21-q23 (del(6q)) and 17p13 (del(17p)).1 Del(13q), associated with a good prognosis, is the site of the microRNA genes, miR-15a/16-1, which negatively regulate BCL2 post-transcriptionally.2 Their deletion results in overexpression of the anti-apoptotic protein BCL2. In contrast,  

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del(17p), the site of TP53, and del(11q), the site of ATM and the miR34b/c cluster, are markers of poor prognosis.\(^1,5\) Cases with +12 have an overall survival (OS) that lies in the middle range between that of cases with del(13q) and those with del(11q) or del(17p).

Although +12 CLL comprises approximately 20% of cases, relatively little is known about its pathophysiology. These cases often demonstrate atypical morphological and immunophenotypic features.\(^4\) Patients with +12 CLL show a higher incidence of thrombocytopenia, Richter transformation, and other secondary cancers, their main cause of death.\(^5\) However, compared to other cytogenetically-defined CLL subtypes, few studies have attempted to identify the critical protein-coding and microRNA genes that are relevant to its pathophysiology.\(^6,9\) For many cancer types, gene dosage correlates strongly with mRNA, microRNA, and protein expression.\(^6,9\) This suggests that at least a subset of the more than 1000 protein-coding genes (and more than 40 microRNA genes) on chromosome 12 in +12 CLL are likely to show a concordant increase in expression. Conceivably, some of these proteins modulate expression of downstream targets, either on chromosome 12 or other chromosomes, resulting in aberrant gene expression.

We aimed to identify protein-coding genes whose expression contributes to the unique pathophysiology of +12 CLL. We performed transcriptional profiling on CLL cases with +12 as the only cytogenetic abnormality, and compared them to cases with only del(13q), del(11q), or diploid cytogenetics. We demonstrate that CLL cases with +12 as the only cytogenetic abnormality express a unique set of activated pathways compared to other cytogenetic subtypes, several of which may represent new therapeutic targets.

### Methods

#### Sample collection and preparation

Between 2000 and 2008, we obtained peripheral blood (PB) from 250 treatment-naïve CLL patients. This study was approved by the Institutional Review Board and conducted according to principles expressed in the Declaration of Helsinki. We extracted nucleic acids from negatively-selected CLL cells (CD19\(^+\)) and prepared cDNA, as described previously.\(^10\) Cases were divided into discovery and validation sets, as described in the Online Supplementary Methods.

#### Single nucleotide polymorphism (SNP) genotyping, IGHV and NOTCH1 mutation status, and ZAP70 protein expression

We assessed genomic copy number variations (CNV) by single nucleotide polymorphism (SNP) genotyping.\(^10\) The IGHV somatic mutation status was assessed as described previously, with minor modifications. Patient and germline sequences were aligned in VBASE II. Cases with <2% mutations compared to germline were designated “unmutated”; cases with ≥2% mutations were designated “mutated”.\(^11\) NOTCH1 exon 34 mutation hotspots were assessed as described previously.\(^12\) We assessed ZAP70 protein expression by immunohistochemistry or flow cytometry.

#### Transcriptional profiling of protein-coding genes

For discovery, we performed transcriptional profiling using gene expression microarrays (Online Supplementary Methods).\(^13\) To identify differentially expressed (DE) genes, we compared the +12 cohort to the others individually and jointly. We assessed DE genes between subtypes by performing probe-by-probe ANOVA (for multiple subtypes) or t-tests (for two subtypes). We fit a beta-uniform-mixture (BUM) model to the set of P-values in order to find the false discovery rate (FDR). Microarray data are available at http://silicovore.com/CLL/Trisomy12. For validation, we performed transcriptional profiling using an MF-QRT-PCR assay to a subset of DE genes identified using microarrays, along with 5 endogenous control genes.\(^13\) We performed gene-by-gene ANOVA and t-tests to validate DE genes discovered from the microarrays. A discovery was considered “validated” if the unadjusted P-value was <0.05.

#### Survival analysis

We performed time-to-event (survival) analysis using Cox proportional hazards models, and assessed significance using the log-rank (score) test. To assess multivariate models, we used a forward-backward stepwise algorithm to eliminate redundant factors and optimize the Akaike Information Criterion (AIC). We performed the computations using the survival package in R v.3.5.0, and computed median follow-up times using the reverse Kaplan-Meier estimator.\(^14\)

#### Pathway analysis

We performed pathway analysis using Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City, CA, USA). The statistical significance of altered pathways was calculated using a one-sided Fisher exact test. The z-score indicates if a pathway is activated (positive) or down-regulated (negative). We also examined ratio values (the number of molecules with expression levels above or below the mean, divided by the total number of molecules in that pathway) for the canonical pathways.

#### Results

**Patients’ characteristics of discovery and validation sets**

Based on SNP genotyping, we divided cases into cytogenetic subsets defined by abnormalities that would be detected using a FISH probe panel to the common CLL cytogenetic abnormalities: del(11q), del(13q), del(17p), +12, and diploid. The proportion of cases in each subset is similar to that reported previously. Because patients were treatment-naïve, there was only one del(17p) case, which we excluded from subsequent analysis; a single case is insufficient to achieve statistical significance in a comparison of gene expression profiles to other cytogenetic subtypes.

We focused our experiments on cases with only +12 compared to cases with only del(13q), del(11q), or diploid cytogenetics. We identified 147 CLL samples that met our inclusion criteria. One hundred and twenty-three contained a single abnormality or were diploid: 27 with +12 as the only abnormality (18%), 49 with del(13q) as the only abnormality (33%), and 47 diploid cases (32%). The discovery set was chosen to contain patients with only abnormalities who later went on to receive front-line therapy with FCR. Because there were relatively few cases with del(11q) as the only abnormality, to achieve statistical significance, we used 10 cases with del(11q) as the only abnormality for the discovery set, but included 14 cases with del(11q) and del(13q) in the validation set. Patients’ characteristics are summarized in Tables 1 and 2.

The discovery set contained no statistically significant
The association between +12 and atypical immunophenotype approached significance ($\chi^2$ test, $P=0.06757$). 4,15 Trisomy 12 cases were more likely to demonstrate unmutated IGHV genes ($P=0.0015$), and express ZAP70 ($P=0.0136$) or CD38 ($P=0.0275$) than del(13q) cases, with statistical significance. The proportion of del(11q) or diploid cases with unmutated IGHV genes and CD38 expression was similar to +12 cases. There was no statistical difference in ZAP70 protein expression between the three groups.

Corresponding to their increased frequency of poor prognostic features (i.e. unmutated IGHV status, CD38 positivity), +12 patients required treatment earlier during their disease course (median, 22.5 months) than del(13q) patients (median, 27.5 months), and at around the same timepoint as diploid patients (median, 23.5 months), but later than sole del(11q) patients (median, 9.6 months) (Figure 1A). All 97 discovery set patients and 22 out of 44 validation set patients subsequently received front-line chemoimmunotherapy with fludarabine, cyclophosphamide, and rituximab (FCR). Trisomy 12 patients had longer progression-free survival (PFS) after treatment (median, >150 months) than patients with del(13q) (median, >150 months). The association between +12 and atypical immunophenotype approach significance ($\chi^2$ test, $P=0.06757$). 4,15 Trisomy 12 cases were more likely to demonstrate unmutated IGHV genes ($P=0.0015$), and express ZAP70 ($P=0.0136$) or CD38 ($P=0.0275$) than del(13q) cases, with statistical significance. The proportion of del(11q) or diploid cases with unmutated IGHV genes and CD38 expression was similar to +12 cases. There was no statistical difference in ZAP70 protein expression between the three groups.

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Table 1. Patients’ characteristics of the discovery set.

|                      | Sole +12 (n=15) | Sole del(13q) (n=40) | Sole del(11q) (n=10) | Diploid (n=32) | Statistic |
|----------------------|-----------------|----------------------|----------------------|----------------|-----------|
| Age at diagnosis (years) | Median 57        | 55                   | 57                   | 52             | $F=2.4008$ |
|                      | Range 51 – 74    | 27 – 70              | 48 – 80              | 34 – 77        | $P=0.0727$ |
| Gender, n (%)        | Male 12 (80%)    | 30 (75%)             | 10 (25%)             | 8 (25%)        | $\chi^2=1.1993$ |
|                      | Female 3 (20%)   | 10 (25%)             | 1 (10%)              | 8 (25%)        | $\chi^2=0.7532$ |
| Rai stage, n (%)     | 0-2 12 (80%)     | 30 (75%)             | 10 (100%)            | 22 (69%)       | $\chi^2=4.2644$ |
|                      | 3-4 3 (20%)      | 10 (25%)             | 0 (0%)               | 10 (31%)       | $P=0.2343$ |
| WBC count, n (%)     | <150 G/L 12 (75%)| 33 (85%)             | 10 (100%)            | 26 (81%)       | $\chi^2=2.3084$ |
|                      | ≥150 G/L 3 (25%) | 6 (15%)              | 0 (0%)               | 6 (19%)        | $P=0.509$ |
| WBC count, (1x109/L) | Median 77        | 110                  | 57                   | 71             | $F=0.8942$ |
|                      | Range 23 – 364   | 9 – 319              | 30 – 135             | 21 – 372       | $P=0.4473$ |
| Prolymphocytes, (% in PB) | Median 6        | 5                    | 3                    | 5              | $F=2.0346$ |
|                      | Range 1 – 22     | 0 – 19               | 0 – 18               | 0 – 13         | $P=0.1147$ |
| β2M, n (%)           | ≥4 mg/L 12 (80%) | 30 (77%)             | 6 (60%)              | 20 (63%)       | $\chi^2=2.9538$ |
|                      | >4 mg/L 3 (10%)  | 9 (23%)              | 4 (40%)              | 12 (37%)       | $P=0.3988$ |
| Immunophenotype, n (%) | Atypical 7 (45%) | 3 (10%)              | 3 (30%)              | 6 (19%)        | $\chi^2=7.5032$ |
|                      | Typical 8 (55%)  | 33 (87%)             | 7 (70%)              | 26 (81%)       | $P=0.0552$ |
| Light chain subtype, n (%) | Kappa 8 (55%) | 17 (45%)            | 8 (80%)              | 24 (75%)       | $\chi^2=6.633$ |
|                      | Lambda 7 (45%)  | 21 (55%)             | 2 (20%)              | 8 (25%)        | $P=0.0346$ |
| IGHV status, n (%)   | Mutated 5 (33%)  | 24 (60%)             | 0 (0%)               | 7 (22%)        | $\chi^2=18.154$ |
|                      | Unmutated 10 (67%)| 16 (40%)            | 10 (100%)            | 25 (78%)       | $P=0.0004$ |
| ZAP70 status, n (%)  | Positive 8 (62%) | 14 (40%)             | 3 (30%)              | 17 (65%)       | $\chi^2=5.4413$ |
|                      | Negative 5 (38%) | 21 (60%)             | 7 (75%)              | 9 (35%)        | $P=0.1422$ |
| CD38 expression, n (%) | ≥30% 5 (33%) | 3 (30%)             | 4 (40%)              | 9 (28%)        | $\chi^2=8.088$ |
|                      | <30% 10 (67%)    | 35 (92%)             | 6 (60%)              | 23 (72%)       | $P=0.0042$ |
| NOTCH1 status, n (%) | Mutated 6 (40%)  | 1 (3%)               | 0 (0%)               | 4 (13%)        | $\chi^2=15.076$ |
|                      | Unmutated 9 (60%)| 34 (97%)             | 10 (100%)            | 26 (87%)       | $P=0.0017$ |

WBC: white blood cell; PB: peripheral blood; β2M: serum β2 microglobulin; n: number *Continuous variables were evaluated with analysis of variance (F-test); categorical variables were evaluated with a χ² test. Values in bold are statistically significant.
an, 61.5 months), del(11q) (median, 62.5 months), or diploid cytogenetics (median, 66.2 months) (Figure 1B). With a median follow up of 146 months (95%CI: 144–157 months) from sample, and 181 months (95%CI: 168–200 months) from diagnosis, we found no statistically significant difference in overall survival (OS). Results for the cytogenetic subsets in the validation and combined datasets were similar.

Trisomy 12 cases have a unique gene expression profile

Next we performed univariate probe-by-probe analysis of variance (ANOVA) and identified 1263 probes representing 1012 unique protein-coding genes, 40 ncRNAs, and 22 ESTs that were differentially expressed between at least two of the four cytogenetic subtypes (FDR=1%; unadjusted \( P = 0.00385 \)). Clustering samples using all 1263 probes showed that the gene expression signature of +12 cases (green) was distinct from the other subtypes (Figure 2). (See Online Supplementary Figure S1 for principal components analysis.) Similarly, most del(13q) cases formed a single cluster (blue), as did the del(11q) cases (pink). In contrast, diploid cases were found in three clusters, admixed with a subset of del(13q) cases. We identified no clinical or laboratory features to account for 4 of the diploid cases clustering with +12 cases. Because the genes selected for this analysis were based on ANOVA, and known only to be different between at least two of the cytogenetic subtypes, it is significant that the +12 cluster is clearly distinct from the other subtypes. Thus, +12 CLL has a unique gene expression profile.

Differences between +12 and del(13q), del(11q), or diploid CLL cases

To identify DE probes/genes between +12 cases and del(13q), del(11q), and diploid cases, respectively, we performed univariate \( t \)-tests. Comparing +12 and del(13q) cases, we identified 1181 DE probes representing 927 unique protein-coding genes, 41 ncRNAs, and 15 ESTs (FDR=1%, unadjusted \( P = 0.000333 \)). Thirty-one (2.6%) of the DE probes represent genes (e.g. BCL2, EIF4B, EIF4E, PIM1) that are known or predicted targets of miR-15a/miR-16-1, within the 13q minimally deleted region (MDR). In addition, 365 out of 1181 (31%) of the DE probes are on chromosome 12; 364 out of 365 are over-expressed in +12 compared to del(13q) CLL. Comparing +12 and del(11q) cases, we identified 756 DE probes representing 585 unique genes, 19 ncRNAs, and 7 ESTs (FDR=5%, unab-

| Table 2. Patients’ characteristics of the validation set. |
|----------------|----------------|----------------|----------------|----------------|----------------|
|                | Sole +12        | Sole del(13q)  | Sole del(11q)  | Diploid        | Statistic      |
|                | (n=12)          | (n=9)          | (n=14)         | (n=15)         |                |
| Age at diagnosis (years) | Median 59 | 60 | 64 | 52 | \( F = 0.4572 \) |
| Range          | 39 – 82 | 38 – 70 | 40 – 81 | 38 – 78 | \( P = 0.6931 \) |
| Gender, n (%)  | Male 7 (58%) | 4 (44%) | 7 (87%) | 7 (47%) | \( \chi^2 = 4.2724 \) |
|                | Female 5 (42%) | 5 (56%) | 1 (13%) | 8 (53%) | \( P = 0.2355 \) |
| Rai stage, n (%) | 0-2 11 (92%) | 7 (78%) | 8 (100%) | 13 (87%) | \( \chi^2 = 2.2464 \) |
|                | 3-4 1 (8%) | 2 (22%) | 0 (0%) | 2 (13%) | \( P = 0.5229 \) |
| WBC count, n (%) | <150 G/L 10 (83%) | 8 (89%) | 7 (87%) | 12 (80%) | \( \chi^2 = 0.4172 \) |
|                | ≥150 G/L 2 (17%) | 1 (11%) | 1 (13%) | 3 (20%) | \( P = 0.9567 \) |
| Prolymphocytes, (%) | Median 73 | 75 | 82 | 75 | \( F = 0.0388 \) |
| Range          | 8 – 364 | 41 – 193 | 35 – 206 | 30 – 209 | \( P = 0.9896 \) |
| \( \beta 2M, n \) (mg/L) | ≤4 mg/L 9 (75%) | 9 (100%) | 5 (63%) | 9 (60%) | \( \chi^2 = 5.0531 \) |
|                | >4 mg/L 3 (25%) | 0 (0%) | 3 (37%) | 6 (40%) | \( P = 0.1679 \) |
| Immunophenotype, n (%) | Atypical 6 (50%) | 3 (43%) | 0 (0%) | 3 (20%) | \( \chi^2 = 7.140 \) |
|                | Typical 6 (50%) | 4 (57%) | 8 (100%) | 12 (80%) | \( P = 0.006757 \) |
| Light chain subtype, n (%) | Kappa 8 (67%) | 4 (57%) | 7 (87%) | 11 (73%) | \( \chi^2 = 1.8725 \) |
|                | Lambda 4 (33%) | 3 (43%) | 1 (13%) | 4 (27%) | \( P = 0.5993 \) |
| \( IGHV \) status, n (%) | Mutated 3 (25%) | 8 (100%) | 1 (13%) | 8 (57%) | \( \chi^2 = 15.432 \) |
|                | Unmutated 9 (75%) | 0 (0%) | 7 (87%) | 7 (43%) | \( P = 0.0015 \) |
| ZAP70 status, n (%) | Positive 4 (44%) | 0 (0%) | 6 (86%) | 7 (54%) | \( \chi^2 = 10.681 \) |
|                | Negative 5 (56%) | 7 (100%) | 1 (14%) | 6 (46%) | \( P = 0.0136 \) |
| CD38 expression, n (%) | ≥30% 5 (42%) | 0 (0%) | 6 (75%) | 5 (33%) | \( \chi^2 = 8.137 \) |
|                | <30% 7 (58%) | 7 (100%) | 2 (25%) | 10 (67%) | \( P = 0.0275 \) |
| NOTCH1 status, n (%) | Mutated NA | NA | NA | NA | |
|                | Unmutated NA | NA | NA | NA | |

WBC: white blood cell count; \( \beta 2M \): \( \beta 2 \) microglobulin; n: number; PB: peripheral blood. *Continuous variables were evaluated with analysis of variance (F-test); categorical variables were evaluated with a \( \chi^2 \) test. Values in bold are statistically significant.
justed P=0.00091). Forty-three (5.9%) of the DE probes represent genes on 11q22.3 (e.g. ATM, NPAT, DDX10, CUL5, ACAT1); all are expressed at higher levels in +12 and at lower levels in del(11q) cases. In addition, 208 out of 736 (28%) of the DE probes are on chromosome 12; 206 out of 208 are over-expressed in +12 compared to del(11q). Comparing +12 and diploid cases, we identified 1229 DE probes representing 964 unique genes, 49 ncRNAs, and 18 ESTs (FDR=5%, unadjusted P=0.00164); 413 out of 1229 (34%) DE probes are on chromosome 12 (χ²=92.5, P<2e-16), and all 413 are over-expressed in +12 compared to diploid.

Consistent differences between +12 and other cytogenetic subtypes

Because the pair-wise DE comparisons included genes whose expression characterizes other cytogenetic subtypes (e.g. BCL2 and other miR-15-a/16-1 targets when comparing +12 to del(13q) cases, or ATM and other genes in the commonly deleted region when comparing +12 to del(11q) cases), we used t-tests to identify DE genes when comparing +12 cases to the union of all other cases in the study. We found 1226 DE probes representing 953 unique genes, 40 ncRNAs, and 17 ESTs (FDR=1%, unadjusted P=0.000347); 419 out of 1226 (34%) were on chromosome 12, and 418 out of 419 were over-expressed in +12 cases. We also observed that 1194 out of 1226 (97%) of these probes were DE in the direct comparison with del(13q); 892 out of 1226 (73%) were DE in the direct comparison to diploid cases, and 526 out of 1226 (43%) were DE in the direct comparison to del(11q). The full list of DE probes is presented in Online Supplementary Table S1.

Among cases with sole +12, microarray profiling data and NOTCH1 mutation status were available for 15 patients in the discovery set: n=9 wild-type (60%), n=6 mutated (40%). To identify DE genes between these subsets, we performed the following analysis. We removed low-expressing probes, and retained a probe only if its expression was >4 on the log2 scale in at least 3 out of 15 samples; 20,776 of the 47,231 probes satisfied this criterion. We performed probe-by-probe t-tests to compare expression between the NOTCH1 mutated versus wild-type samples. We found 389 DE genes with P<0.01 (FDR=45%; Online Supplementary Table S2).

Pathway analysis

We performed Ingenuity Pathway Analysis (IPA) to identify differentially regulated canonical pathways that distinguish +12 from other cytogenetic subtypes individually. For del(13q), del(11q), and diploid subtypes we performed analyses using the 1181, the 736, and the 1229 DE probes described above. For each comparison, ten pathways were identified as either activated or down-regulated with the most statistical significance, based on the Ingenuity z-scores (Table 3). Complete data are listed in Online Supplementary Table S3.

Validation of potential targets

To validate potential mRNA targets identified by whole-genome transcriptional profiling performed on the discovery set, we assessed expression of a subset of these genes by MF-QRT-PCR assay on an independent validation set of 50 patient samples. We used the QRT-PCR assay because it is more reproducible and has a wider dynamic range than microarray profiling. Of 135 genes assayed, 64 (47%) were fully validated for all comparisons between +12 and other pure cytogenetic subsets, and 91 (67%) were validated for at least half of the comparisons. A subset of 31 genes assayed using the MF-QRT-PCR assay are included in the network diagram in Figure 3; of these, 19 (61%) were fully validated and all 31 (100%) were validated in at last half of the comparisons of +12 with other cytogenetic groups. Complete data are listed in Online Supplementary Table S4.

A +12 specific network

Using the MF-QRT-PCR data, we constructed a gene network whose expression in +12 cases differed from other subtypes. We selected all DE genes with FDR=5% (unadjusted P=0.0016) and fold change (FC) ≥2 in comparison to both del(13q) and diploid cases. Intersecting these lists yielded 109 probes that represent 92 distinct genes. Although 17 probes did not satisfy the criteria when we compared +12 cases directly to del(11q), we chose to retain them because all 109 probes satisfied the selection criteria.

Figure 1. Kaplan Meier plots stratified by cytogenetic subtype. (A) Time to treatment, and (B) progression-free survival.
criteria in the analysis that compared +12 cases to all other samples. Starting with these 92 genes, we used the network building tools in IPA. First, the “connect” operation joins any pair of genes whose interaction is supported in the literature. Then the “grow” operation adds genes from the literature (not from the initial list) that are significantly connected to genes already in the network. We performed the “connect” and “grow” operations twice; the resulting network is shown in Figure 3. Genes not connected to the main network and not on chromosome 12 were omitted from the final diagram.

Discussion

Despite important differences in the clinical and pathophysiological features of +12 CLL compared to other cytogenetic subtypes, only a few transcriptional profiling studies (some on small numbers of samples) have focused on identifying dysregulated pathways that characterize +12 CLL and that may serve as therapeutic targets.7,8,18 Furthermore, these studies have classified cases using a hierarchical system, i.e. cases with more than one recurrent abnormality are categorized according to the abnormality with the poorest prognosis.7,8,18 Thus, cases with both del(13q) and +12 are classified as +12, and cases with both +12 and del(11q) are classified as del(11q). To exclude the likely confounding effects of multiple cytogenetic abnormalities on gene expression, our +12 patient cohort had +12 as the only abnormality.

Similar to previous studies, we found that patients with +12 and with diploid cytogenetics required treatment earlier during their disease course than patients with del(13q), but later than patients with del(11q). Following FCR chemoimmunotherapy, our +12 cohort had a longer PFS than patients with other cytogenetic subtypes, but showed no difference in OS. Thus, +12 CLL patients may require treatment earlier, but respond better to FCR, than patients with other cytogenetic abnormalities. Higher CD20 expression by +12 CLL compared to other cytogenetic subtypes may account, in part, for the high rate of response to rituximab-based therapy.19 However, despite the good response of +12 CLL patients to FCR, it is poorly tolerated by unfit patients or those over 65 years of age. Some patients develop myelosuppression and neutropenic fevers, and cannot receive a full course of therapy. Finally, a small percentage of patients treated with alkylating agents and fludarabine develop secondary myeloid malignancies.20 Thus, there is a need for less toxic, targeted therapies.

As expected, we identified genes whose expression patterns are known to be associated with cytogenetic subtypes, giving us confidence in our methods. For example, we identified statistically significant differences in gene expression between +12 cases with and without NOTCH1 mutation. Although the number of cases is rel-
Table 3. Ingenuity Pathway Analysis of differentially-regulated canonical pathways.

| Pathway | +12 vs. del(13q) | +12 vs. del(11q) | +12 vs. diploid | +12 vs. all |
|---------|-----------------|-----------------|----------------|------------|
| Phospholipase C signaling | A | TNFR2 signaling | D | Ceramide signaling | D | Protein kinase A signaling | A |
| Integrin signaling | A | HIPPO signaling | A | Non-small cell lung cancer signaling | A | Integrin signaling | A |
| Regulation of actin-based motility by Rho | A | CD40 signaling | A | NGF signaling | A | Phospholipase C signaling | A |
| Remodeling of epithelial adherens junctions | A | Death receptor signaling | D | Integrin signaling | A | Ceramide signaling | A |
| Protein kinase A signaling | A | TWEAK signaling | D | Pancreatic adenocarcinoma | D | Cell cycle: G1/S checkpoint regulation | D |
| Role of BRCA1 in DNA damage response | D | Sphingosine-1-phosphate signaling | A | Huntington disease signaling | A | Role of BRCA1 in DNA damage response | D |
| RhoGDI signaling | D | TNFR1 signaling | D | Glioma signaling | A | Insulin receptor signaling | A |
| Fcγ receptor-mediated phagocytosis in macrophages and monocytes | A | Signaling by Rho family GTPases | A | H3-3-mediated signaling | D | Huntington disease signaling | A |
| Non-small cell lung cancer signaling | A | Regulation of actin-based motility by Rho | A | IL-8 signaling | A | Remodeling of epithelial adherens junctions | D |
| Gqα signaling | A | RhoGDI signaling | D | HMGB1 signaling | A | Glioma signaling | A |

vs.: versus; S: Status of pathway compared to +12 CLL; A: activated; D: down-regulated.

Atively small, our results are similar to those of previous studies.24,25 However, we found no differences in expression of NOTCH1 or NOTCH1 target genes (e.g. HES1, DTX1, NRARP) between NOTCH1-mutated and unmutated cases. Recently, Fabbi et al. analyzed NOTCH1 RNA expression in normal B-cell subsets and more than 100 PB CLL cases.24 They found that tonsillar naïve and memory B cells expressed NOTCH1, HES1, and MYC, while germinal center B cells were negative. Furthermore, about 50% of CLL cases that lacked NOTCH1 mutations expressed the active intracellular portion of NOTCH1. They describe a “NOTCH1 gene expression signature” that regulates critical B-cell processes, but is independent of NOTCH1 mutation.

Using pathway analysis, we identified canonical pathways that are differentially regulated in +12 CLL compared to other subtypes; several converge on the BCR signaling pathway. One of the most highly activated pathways was integrin signaling. Integrins are transmembrane receptors that mediate interactions between the extracellular matrix and actin cytoskeleton. Integrins enhance adhesion, which activates signaling pathways that regulate migration, proliferation, cell survival, and other processes.26 B-cell receptor signaling is critical for CLL survival and proliferation,27 and is enhanced through interactions between CLL cells and the microenvironment.28 Signals from the BCR are transduced by downstream kinases. Therapeutic agents that target Bruton’s tyrosine kinase (BTK) and the phosphorylatedinositol-4,5-bisphosphate 3-kinase catalytic subunit delta (PI3Kδ) are highly effective.29 Patients treated with kinase inhibitors experience a rapid rise in absolute lymphocyte count (ALC) due to egress of CLL cells from lymph nodes that declines slowly over approximately eight months.27-29 The magnitude and duration of this transient redistribution lymphocytosis varies with cytogenetic subtype; del(13q) patients with IGHV-mutated genes tend to experience prolonged lymphocytosis, while +12 patients show an attenuated rise in the ALC and a more rapid reduction to baseline.30,31 Redistribution lymphocytosis is likely a consequence of inhibition of CLL migration and adhesion due to disruption of chemokine receptor and integrin signaling. For example, the BTK inhibitor ibrutinib abrogates adhesion of CLL cells to fibronectin, interfering with their ability to adhere to stromal cells.31 Recent studies indicate that +12 CLL express higher levels of several integrin proteins compared to other cytogenetic subtypes.25,26 Similarly, we found overexpression of ITGAL and ITGB2 (which encode the αL and β2 chains of LFA-1), ITGA4 (which encodes the α4 chain of VLA-4), and ITGB7. We also observed overexpression of ITGB5 and vinculin (VCL). ITGB5 encodes the β5 integrin chain, a fibronectin receptor component. Increased ITGB5 may contribute to the attenuated redistributive lymphocytosis in +12 CLL by increasing adhesion to fibronectin and interfering with the CLL egress from lymph nodes. Vinculin stabilizes integrins at the immune synapse, the interface between the B cell and the antigen-presenting cell, and is critical for activation of BCR signaling.32 Thus, increased integrin-mediated signaling may promote retention of +12 CLL cells within tissues. Alternatively, circulating +12 CLL cells may undergo more rapid apoptosis...
due to greater reliance on microenvironmental survival signals, another factor that may contribute to their good response to FCR.30

We identified three NFAT mRNAs, NFATc1 (NFAT2), NFATc2 (NFAT1), and NFATc3 (NFAT4), that are overexpressed in +12 CLL, and involved in three activated signaling pathways: protein kinase A, phospholipase C, and integrin signaling. The NFAT (nuclear factor of activated T cells) transcription factor family contains five proteins; four are regulated by calcium and the calcineurin signaling pathway.34,35 Originally described in T cells, NFAT proteins are expressed by B cells, natural killer cells, and other cell types. They regulate genes involved in cell cycle, apoptosis, angiogenesis, and metastasis.

In resting lymphocytes, inactive hyperphosphorylated NFAT proteins are confined to the cytoplasm.34,35 In B cells, BCR ligation by cognate antigen activates SYK, which phosphorylates and activates BTK.36 BTK then phosphorylates and activates PLCg2, which catalyzes the hydrolysis of inositol 1, 4, 5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 mediates influx of extracellular calcium and calcium release from intracellular stores, which results in calcium/calmodulin-dependent activation of calcineurin. In the cytoplasm, calcineurin cleaves phosphate groups from inactive, hyperphosphorylated NFAT proteins, which enter the nucleus, bind to specific response elements in target gene promoters (alone or in combination with partner proteins), and activate or inhibit transcription. Thus, NFAT proteins integrate calcium signaling with other signaling pathways, including the MAPKinase, WNT, and NOTCH pathways.

Dysregulated calcineurin/NFAT signaling has been reported in carcinomas and lymphoid malignancies. In large B-cell lymphomas, active NFAT interacts with NF-κB and directly regulates CD154 expression to maintain growth.37 Despite its central role in BCR signaling, there are few studies of NFAT signaling in CLL.38,39 LeRoy et al. demonstrated that BCR-NFAT signaling affects CLL clinical outcome, and suggest that BCR-NFAT intermediates serve as therapeutic targets.38 Recently, Oakes et al. compared the epigenetic programming of normal B-cell subsets with 268 CLL samples, and identified aberrant NFAT methylation in a CLL subset.40 Thus, efforts are underway to develop more specific NFAT inhibitors.33,44

Using IPA to construct a novel +12 specific network, we identified ecto-5'-nucleotidase (NT5E, CD73) as an important element. NT5E, an immune checkpoint molecule of potential therapeutic value, is expressed in a wide variety of tissues.45-47 Immune checkpoint molecules regulate interactions between immune and tumor cells, and may stimulate or inhibit these interactions.45,46 Many cancers exploit these molecules to evade an anti-tumor immune response. Among the best described are the inhibitory molecules PD1, PD-L1, and CTLA-4. CLL is characterized by immunosuppression and an inefficient anti-tumor response that results from defects in humoral and cellular immunity, including ineffective T-cell responses and expression of exhaustion-like surface markers, such as PD-L1.48-50 Immune checkpoint inhibitors that target the PD1/PD-L1 and CTLA-4 pathways are being used to treat a variety of tumors, including melanoma and prostate cancer.

NT5E catalyzes the conversion of extracellular ATP to adenosine, which is critical for immune function.45,46 Among immune cells, it is expressed in macrophages, B cells, regulatory T cells, and dendritic cells. NT5E helps tumors evade the immune response by inhibiting the activation, proliferation, and homing of tumor-specific T cells, and by enhancing conversion of anti-tumor type 1 macrophages to pro-tumor type 2 macrophages. The NT5E-adenosine axis constitutes a promising new pathway in cancer immunotherapy. Targeted blockade of NT5E or adenosine receptors promotes anti-tumor immunity and enhances the activity of first-generation immune checkpoint blockers.46,47 Phase I clinical trials evaluating the efficacy of anti-NT5E or anti-A2A therapies in cancer patients are underway. However, few studies have investigated the functions of NT5E in lymphoid malignancies. In a study of CLL patients, 30% of cases expressed NT5E,
which was associated with aggressive disease, and CD38 and ZAP70 positivity. Unfortunately, this study did not assess the association between NT5E expression and cytogenetic status. Our findings suggest that targeting this pathway may be an effective therapy in patients with +12 CLL.

In summary, we have demonstrated, by whole transcriptome profiling, that CLL cases with +12 as the only cytogenetic abnormality demonstrate a unique set of differentially expressed genes and pathways compared to cases with del(13q) or del(11q). Our data support the hypothesis that these differences contribute, in part, to the unique pathophysiology of +12 CLL. Finally, we have identified genes and pathways, such as the checkpoint inhibitor molecule, NT5E (CD73), and the NFAT signaling pathway that may represent new therapeutic targets.

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