

Early Changes in Gene Expression Profiles in AML Patients During Induction Chemotherapy

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Research Article

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Abstract

Background: Elucidation of the genetic mechanisms underlying treatment response to standard induction chemotherapy in AML patients is warranted, in order to aid in risk-adapted treatment decisions as novel treatments are emerging. In this pilot study, we explored the treatment-induced expression patterns in a small cohort of AML patients by analyzing differential gene expression (DGE) over the first two days of induction chemotherapy.

Methods: Blood samples were collected from ten AML patients at baseline (before treatment initiation) and during the first two days of treatment (Day 1; approximately 24 h, and Day 2; approximately 48 h after treatment initiation, respectively) and RNA was extracted for subsequent RNA sequencing. DGE between time points were assessed by pairwise analysis using the R package edgeR version 3.18.1 in all patients as well as in relation to treatment response (complete remission, CR, vs non-complete remission, nCR). Ingenuity Pathway Analysis (Qiagen) software was used for pathway analysis and visualization.

Results: After initial data quality control, two patients was excluded from further analysis, resulting in a final cohort of eight patients with data from all three timepoints. DGE analysis demonstrated activation of pathways with genes directly or indirectly associated with NF- κ B signaling. Significant activation of the *NF- κ B pathway* was seen in 50% of the patients two days after treatment start, while *iNOS pathway* effects could be identified already after one day. nCR patients displayed activation of pathways associated with cell cycle progression, oncogenesis and anti-apoptotic behavior, including the *STAT3 pathway* and *Salvage pathways of pyrimidine ribonucleotides*. Notably, a significant induction of cytidine deaminase, an enzyme responsible for the deamination of Ara-C, could be observed between baseline and Day 2 in the nCR patients but not in patients achieving CR.

Conclusions: In conclusion, we show that time-course analysis of gene expression represents a feasible approach to identify relevant pathways affected by standard induction chemotherapy in AML patients. This poses as a potential method for elucidating new drug targets and biomarkers for categorizing disease aggressiveness and evaluating treatment response. However, more studies on larger cohorts are warranted to elucidate the transcriptional basis for drug response.

Background

The standard induction chemotherapy in acute myeloid leukemia (AML) have remained roughly the same over the last decades, with treatment consisting of the nucleoside analog cytarabine (Ara-C) in combination with an anthracycline, most commonly daunorubicin or idarubicin. Additional drugs have been used together with the cornerstone drugs in different clinical trials, and in specific cases such as that of acute promyelocytic leukemia, treatments targeting specific chromosomal rearrangements are available (1). However, AML patients still suffer from a significant degree of therapy-related mortality and treatment failure. More knowledge is needed of the genetic mechanisms underlying poor treatment response in AML, in order to achieve better risk stratification and individual adaption of treatment regimens as newer drugs are emerging in clinical trials. Genetic variation in genes encoding drug-metabolizing enzymes, transporters, DNA-repair systems or in genes regulating cell cycle progression and apoptosis is likely influencing cellular drug response both in leukemic and non-leukemic cells, although the clinical utility of such markers in relation to AML treatment decisions remain controversial (2). In leukemia, gene expression profiling of diagnostic samples has been implicated as a tool able to discriminate between different types (e.g ALL and AML) (3) as well as molecular and prognostic subclasses of AML (4–6). It has also been shown that acute lymphoblastic leukemia cells of different molecular subtypes share common but treatment-specific acute gene expression changes induced by mono- or combination therapy, as measured by gene expression arrays of samples collected before and one day after treatment (7). In addition, a recent study of normal p53-regulated apoptosis, induced by DNA damage, demonstrated that the extent of the apoptotic response was attributable to

the rapidity of the transcriptional response rather than to a specific set of target genes (8). This shows that time series analysis of gene expression could provide new insights to the mechanisms behind drug response. The early drug-induced gene expression changes in AML patients undergoing standard induction chemotherapy has, to our knowledge, not been investigated previously. In this pilot study, we aimed to explore the expression patterns in a small cohort of AML patients and analyzing differential gene expression over the first two days of induction chemotherapy. Using RNA sequencing data, the changes in gene expression over time was analyzed overall to identify common patterns, but also compared between patients achieving complete remission and those who did not, to identify potential genes and pathways that might be used to predict or understand the response.

Materials And Methods

Patients and sample collection

10 patients with AML scheduled for induction chemotherapy at the hematology clinic of Linköping University Hospital were included in the study. The study was performed in compliance with the Declaration of Helsinki under the approval by the local ethics committee (Forsknings etikkomittén vid Hälsouniversitet Linköping University, Sweden), and all patients gave informed consent. Patient characteristics and treatment details are summarized in Table 1. Blood samples were collected at baseline (before treatment initiation) and during the first two days of treatment (Day 1; approximately 24 h after treatment initiation, and Day 2; approximately 48 h after treatment initiation). All patients received Ara-C either as 100mg/m² as a continuous infusion (Patient 2) and Patient 3) or as 1g/m² twice daily as 2 h infusions before the Day 1 sample collection; before the Day 2 sample collection all patients except Patient 2 and Patient 3 had also received anthracycline treatment either as idarubicin (10mg/m² as 1 h infusion; Patient 1 and Patient 4) or daunorubicin (60mg/m² as 8 h infusion). Patient 4 was also part of a randomized phase 2 trial investigating the addition of cladribine, as a 5mg/m² 1 h infusion two times, to Ara-C and idarubicin (9). Patient 5 was initially suspected to have acute promyelocyte leukemia and received treatment with all-trans retinoic acid (ATRA) before the final AML diagnosis and inclusion in this study. In addition to chemotherapy, the patients received standard supportive care with common co-medications including dexamethasone eye drops, allopurinol and antiemetics. RNA was extracted from EDTA blood samples using the Qiagen Blood RNA mini kit (Qiagen), or from blood samples stabilized in PAXgene blood RNA tubes (PreAnalytiX) using the PAXgene blood RNA kit (PreAnalytiX), according to the manufacturers' instructions. The RNA concentration was measured on a NanoDrop (Thermo Scientific) and the RNA integrity number (RIN) was assessed using an Agilent 2100 BioAnalyzer (Agilent) with the Agilent RNA 6000 nano kit (Agilent) according to the manufacturer's instructions.

Sequencing and data preprocessing

All samples with a RIN > 7.5 were sent to the National Genomics Infrastructure (NGI, Stockholm, Sweden) hosted by the Science for Life Laboratory, for RNA sequencing. Libraries were prepared with the TruSeq RNA Library Prep Kit v2 (Illumina) with poly-A selection and paired-end (2x100bp) sequencing was performed on the NovaSeq 6000 Sequencing System (Illumina) using an s1 flow cell. Two samples, Patient 6 Day 2 and Patient 10 Day 2, failed library preparation and due to the low amount of extracted RNA no further attempts could be made. Preprocessing of the raw sequencing data was performed by NGI according to the nf-core/rnaseq pipeline (see <https://github.com/nf-core/rnaseq> for details). Briefly, the pipeline processes the raw fastq data using FastQC for read quality control and TrimGalore! for adapter removal and quality trimming. Alignment of the reads to the human reference genome GRCh38 was made using STAR version 2.7.2b (10). The read distributions over genomic features were summarized using featureCounts from the Subread package version 1.6.2 (11, 12), and additional quality-control of the results was performed using DupRadar (13) for duplication rate quality control, edgeR (14, 15) for sample similarity, Preseq (16) for library complexity estimation, and

RSeQC (17) or read distribution, duplication and gene body coverage. Finally, the quality measures were summarized using MultiQC version 1.7 (18).

Differential gene expression analysis

To explore changes in gene expression in response to treatment, R version 3.6.1 (19) and the R package edgeR version 3.18.1 (14, 15) was used. The data was structured to contain read counts, ensemble gene ID, patient ID and sample time point, for all genes with read counts > 1 count per million (CPM) in ≥ 3 samples. The data was then normalized using the calcNormFactors to scale the raw library sizes for each sample. Differential gene expression (DGE) compared to baseline levels were investigated by pairwise comparisons. In the analysis of DGE between time points, where replicates were lacking for individual patients, the biological coefficient of variation (BCV) was defined as 0.4 as recommended in the edgeR user guide. Negative binomial generalized linear models (GLMs) were fitted for each patient using the function GLMFit with the dispersion defined as the BCV squared. Likelihood ratio tests were then performed with the function glmLRT to determine which genes were differentially expressed between the time points. For the overall analysis, the function estimateDisp was used to determine the trended, tagwise and common dispersion for the samples. These were then fitted using glmQLFit and a quasi-likelihood F-test was performed using glmQLFTest. Gene expression changes were also investigated in the clinical response groups, morphological complete remission (CR; n = 5) and non-complete remission (nCR; n = 5) as assessed by bone marrow sampling between day 15 and day 28 after treatment initiation.

Pathway analysis

The function TopTags in edgeR was used to extract differentially expressed (DE) genes and their \log_2 (fold change), p-value and false discovery rate adjusted p-value. DE genes with p-values ≤ 0.05 were used as input for Ingenuity Pathway Analysis (IPA) (QIAGEN) software that analyses and visualizes DE genes to identify activated or inhibited pathways (20). Canonical pathways extracted from IPA with a $-\log(p\text{-value}) \geq 1.3$ (corresponding to a p-value < 0.05) were marked as inhibited if the Z-score was ≤ -2 and activated if it was ≥ 2 . All pathway images in the manuscript were generated using IPA (QIAGEN). Molecules are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least one literature reference or canonical information stored in the IPA knowledge base. The intensity of the node color indicates the degree of up- or down-regulation in red and green, respectively. Nodes are displayed using various shapes that represent the functional class of the gene product. Edges are displayed with various labels that describe the nature of the relationship between the nodes (e.g., P for phosphorylation, T for transcription).

Table 1
AML patient characteristics.

Patient ID	Year of diagnosis	Age	Induction treatment regime	Cytogenetics	<i>FLT3</i> -ITD	<i>NPM1</i> mutation	<i>CEBPA</i> mutation	Morphological complete remission after induction ³
1	2001	67	Idarubicin and cytarabine	Aberrant expression of T-cell markers	NA	NA	NA	No
2	2001	81	Daunorubicin and cytarabine ¹	NA	NA	NA	NA	Yes
3	2003	39	Daunorubicin and cytarabine ¹	Normal karyotype	No	No	NA	Yes
4	2001	67	Cladribine, cytarabine and idarubicin	Trisomy 8	NA	NA	NA	Yes
5	2003	66	Daunorubicin and low-dose cytarabine ²	1-40dmin[22] /46XY[3]	Yes	No	NA	No
6	2015	64	Daunorubicin and cytarabine	Normal karyotype	No	Yes	Heterozygous	Yes
7	2015	74	Daunorubicin and cytarabine	Not possible due to no mitoses after culturing	NA	NA	NA	No
8	2015	73	Daunorubicin and cytarabine	Normal karyotype	Yes	No	No	No
9	2016	21	Daunorubicin and cytarabine	47XY, inv(16)(p13;q22), +mar[23]	No	No	NA	Yes
10	2016	46	Daunorubicin and cytarabine	Complex karyotype	No	No	No	No

¹Initially only cytarabine; no daunorubicin during study sample collection. ²After one previous day of all-trans-retinoic acid (ATRA) treatment. ³Bone marrow evaluated at day 15–28 after the start of induction chemotherapy.

Results

Sequencing data quality assessment

Based on high duplication rate, skewed GC distribution and large portion of multimapping reads, Patient 6 and 10 were excluded from further analysis. The skewed GC distribution and high levels of overrepresented sequences are indicative

of ribosomal, bacterial or viral RNA contamination, or biased selection during sample preparation. It could be noted that both these patients had extremely high expression of one single gene – *HBB* – encoding the hemoglobin subunit β -hemoglobin, with feature counts of several million. However, due to the overall quality metrics divergences, the decision to exclude these two patients remained. This resulted in a final cohort of eight patients with data from all three time points available.

Common changes in gene expression

Pairwise comparisons with the assessment of overlap between individual patients, and analysis of DGE overall between the time points of the whole cohort demonstrated significant activation of pathways with genes directly or indirectly associated with NF- κ B signaling. This includes processes regulating immune responses, including inflammatory processes, proteasomal processing, lymphogenesis and cell survival as well as proliferation (21). In addition, iNOS production is associated with NF- κ B signaling, and this could be observed in our data set as DGE between time points in the *iNOS pathway* (Fig. 1 and Fig. 2 illustrate the significant transcriptional changes observed in the *NF- κ B pathway* and *iNOS pathway*, respectively). The entire list of affected pathways with Z-scores can be found in **Additional file 1, Table S1** (individual patient data) and **Additional file 2, Table S2** (response groups and Overall data). Significant activation of the *NF- κ B pathway* was seen in 50% of the patients two days after treatment start, while *iNOS pathway* effects could be identified already after one day.

Pathways affected in relation to treatment outcome

We also investigated which pathways were affected during the treatment in relation to patient outcome; CR or nCR, based on day 15 or day 28 bone marrow evaluation. In patients reaching CR, the pathway *Oxidative phosphorylation* was activated already after the first treatment day, while the pathways *Purine de novo biosynthesis II*, *tRNA charging*, and *Cyclins and cell cycle regulation* were inhibited. These pathways were not significant in patients that did not reach CR. In contrast, nCR patients displayed activation of pathways associated with cell cycle progression, oncogenesis and anti-apoptotic behavior, including activation of the *STAT3 pathway* (Fig. 3) and *Salvage pathways of pyrimidine ribonucleotides* (Fig. 4). Notably, a significant induction of cytidine deaminase, an enzyme responsible for the deamination of Ara-C, could be observed between baseline and Day 2 in the nCR patients but not in the patients achieving CR. All pathways affected with their corresponding z-scores in the two response groups are listed in **Additional file 2, Table S2**.

Discussion

In this pilot study, we analyzed gene expression changes over the first two days of standard induction chemotherapy in AML patients. We could show induction of relevant pathways related to immune response, cell proliferation and apoptosis, but also including genes of importance for drug pharmacokinetics. Some of these changes were detectable already after the first day of treatment. Overall, the *NF- κ B pathway* was significantly activated after two days of treatment compared to baseline. This was also reflected by expression alterations in the *iNOS pathway*, which is directly linked to NF- κ B signaling. The canonical NF- κ B pathway is normally activated by pro-inflammatory cytokines, Toll-like receptor binding or T-cell receptor activation, subsequently leading to proteasomal degradation of the NF- κ B inhibitor I κ B. The release from I κ B allows NF- κ B to relocate from the cytoplasm to the nucleus where it regulates a wide variety of genes. In oncogenesis, this includes the transcription of genes promoting cell proliferation and survival as well as angiogenesis, metastasis, and suppression of the tumorsuppressors p53 and FOXO3a (21). In AML, a growing body of evidence has demonstrated dysregulated NF- κ B (22) and opened up the possibility of targeting this pathway; here, proteasome inhibitors are constituting a promising group of drugs with broad application to many types of cancers. In addition, *in vitro* experiments have shown that treatment with cytarabine induces NF- κ B expression in the HL-60 cell line (23). Our results in contrast show relative up-regulation of I κ B and correspondingly down-regulation of NF- κ B between

baseline and Day 2. This is indicative of an overall positive effect of the chemotherapy treatment, inhibiting cell proliferation and survival; however, we cannot determine whether these transcriptional changes are present in all cell populations (malignant as well as healthy) or to which extent these relative changes are reflecting a decreasing proportion of malignant cells (with assumed dysregulated NF- κ B signaling).

When analyzing the patients based on their first response evaluation (bone marrow analysis at day 15 or 28), we were able to identify pathways that were significantly affected in one group but not the other. These differences could potentially be indicative of differences in tumor cell sensitivity to drug treatment. As such, the pathways *Purine de novo biosynthesis II*, *tRNA charging*, and *Cyclins and cell cycle regulation* were inhibited in patients reaching CR already after the first course of induction therapy, which might demonstrate reduced tumor cell proliferation in this group. These pathways were not significantly affected in the group of patients that did not achieve complete remission; however, two patients did so after additional treatment. In contrast to the CR group, nCR patients displayed significant activation of pathways associated with cell cycle progression, anti-apoptotic signaling and malignant transformation. Persistent activation of Stat3, as well as other Stat proteins, has been implicated as an oncogene promoting malignant transformation in various experimental settings (24, 25). Stat3 target genes include Bcl-X and Bcl-2, both encoding anti-apoptotic proteins, and cooperation with c-Jun constitute yet another mechanism of interference with tumor cell apoptosis. In addition, the targets Cyclin D1 and Myc promote proliferation; in solid tumors an influence of Stat3 on VEGF expression could also further promote tumor growth and metastasis/tissue invasion through increased angiogenesis, a process of lower relevance for blood cancers. Many other target genes are likely involved in the oncogenic potential of Stat3, and activation of this protein does not necessarily lead to malignant transformation. However, suppression of Stat signaling has demonstrated tumor growth arrest, induction of apoptosis in cancer cells and inhibition of malignant transformation, indicating that it has potential as a future drug target (24, 26–28). Our results suggest that patients displaying rapid activation of the Stat3 pathway could be harboring more resistant or highly proliferative leukemic clones. Interestingly, another pathway identified to be significantly activated in patients with poor initial treatment response was *Salvage pathways of pyrimidine ribonucleotides*. This pathway represents an important system for the maintenance of ribonucleotide pools in all proliferating cells, but also have implications for drug response (29, 30). Notably, the induction of the enzyme cytidine deaminase (CDA) could result in a more treatment-resistant phenotype in these patients, as the pyrimidine analog Ara-C is subject to degradation into Ara-U by CDA. CDA induction as a mechanism of resistance to Ara-C has been demonstrated *in vitro* (31, 32), and our results support also an *in vivo* relevance.

Some considerations of this study need to be addressed. Even though our study population is small and the influence of interindividual variation may occlude common changes in gene expression related to the treatment, we are partly compensating for this by comparing the different time point data to that from the baseline sample for each patient. However, the bulk RNA sequencing data represent the gene expression in both healthy and cancerous blood cells, and the leukemic blast proportion in blood samples from AML patients varies. Throughout the treatment, blood leukocyte counts are rapidly decreasing in many patients, and in our approach, we are not discriminating between cancer cells and healthy cells being eradicated. Changes in the relative abundance of different cell populations are likely to be reflected in the expression data. As an example, we cannot determine whether a decreased gene expression at day two compared to baseline means that the expression has been inhibited, or if it means that a specific cell population responsible for the baseline expression was eradicated, leaving only cell populations with another pattern. In addition, our patient samples were collected over a long period, and variations in treatment protocols and sampling procedures may have influenced the result. Initial principal component analysis indicated that the data tended to partly cluster depending on inclusion period (defined as before or after the introduction of national treatment guidelines, data not shown) although there was an overlap between these clusters.

In conclusion, we show that time-course analysis of gene expression represents a feasible approach to identify relevant pathways affected by standard induction chemotherapy in AML patients. This poses as a potential method for elucidating new drug targets and biomarkers for categorizing disease aggressiveness and evaluating treatment response. However, more studies on larger cohorts are warranted to elucidate the transcriptional basis for drug response. As single-cell RNA sequencing methodologies are developed and becoming more common, this would likely represent an option to further enhance the resolution also in time course studies like this one, although single-cell approaches are associated with other practical challenges in both sample management and analysis compared to conventional bulk RNA sequencing. Preferably, future studies would include more time points as well as follow-up analysis in the event of relapse.

Declarations

Ethics approval

The study was performed in compliance with the Declaration of Helsinki under the approval by the local ethics committee (Forskningsetikommittén vid Hälsouniversitet Linköping University, registration numbers 98319, 02-459 and 2017-397-31), and all patients gave informed consent to study participation and publication of the study results.

Data availability

The sequencing datasets generated and/or analysed during the current study are not publicly available due to restraints of the ethical permit, but are available from the corresponding author on reasonable request.

Declaration of conflicts of interest

The authors declare no competing interests. The research grant providers had no role in the study design, collection, analyses or interpretation of data, writing of the manuscript, or in the decision to publish the results.

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Authors' contributions

HG: conception and study design, sample preparation and analysis; IJ: sample preparation, clinical data compilation, results interpretation, and manuscript preparation; MS and NB: bioinformatics and statistical analysis, manuscript preparation; KL: Patient recruitment, sample collection and clinical data collection. All authors critically reviewed the manuscript and approved the final version for publication.

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Figures

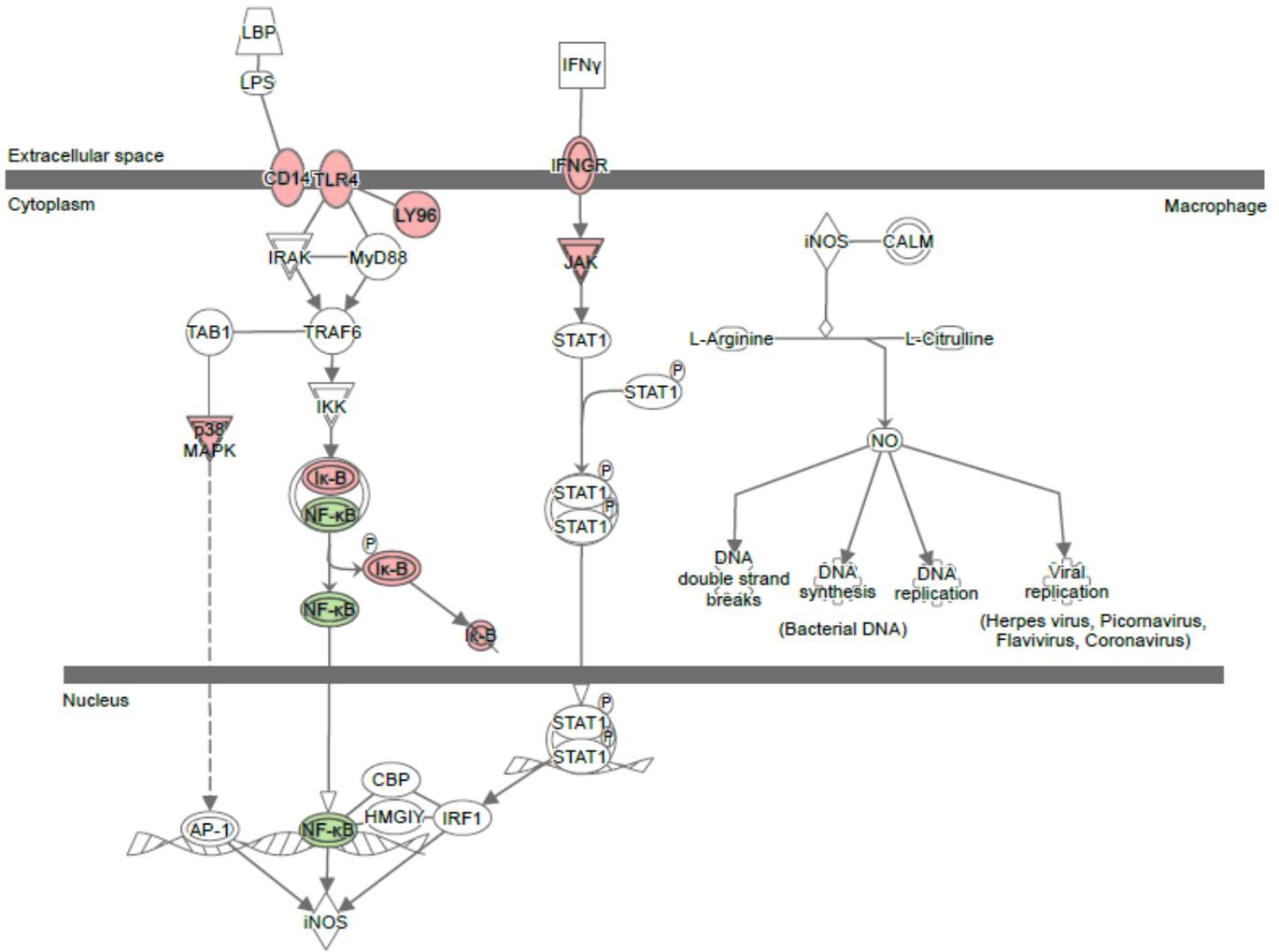


Figure 2

Activation of the iNOS pathway. Genes marked in red are upregulated between baseline and Day 2 while those marked in green are downregulated.

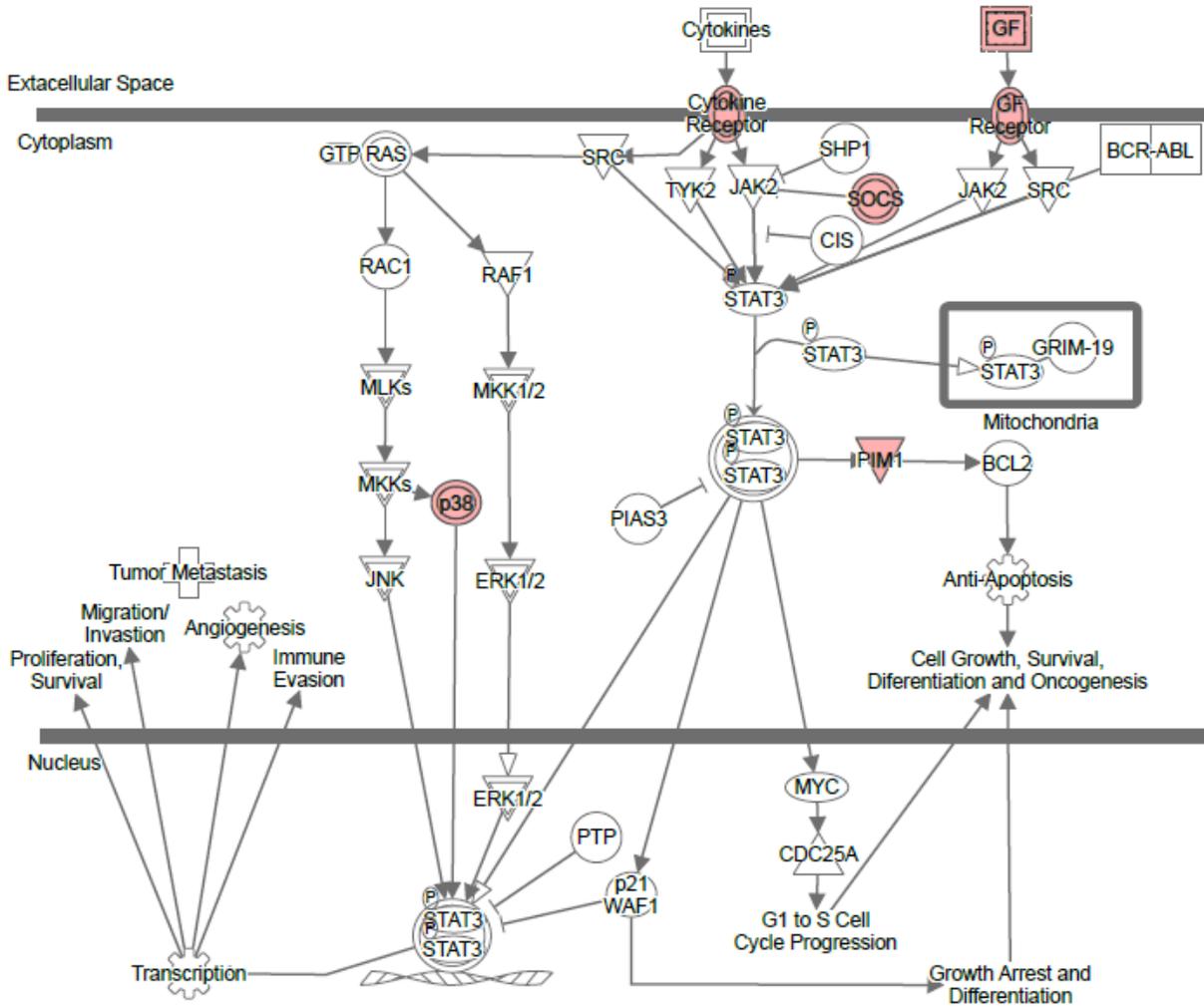


Figure 3

The STAT3 pathway. Genes marked in red are upregulated between baseline and Day 2, in patients that did not reach complete remission (CR) after the first course of induction chemotherapy.

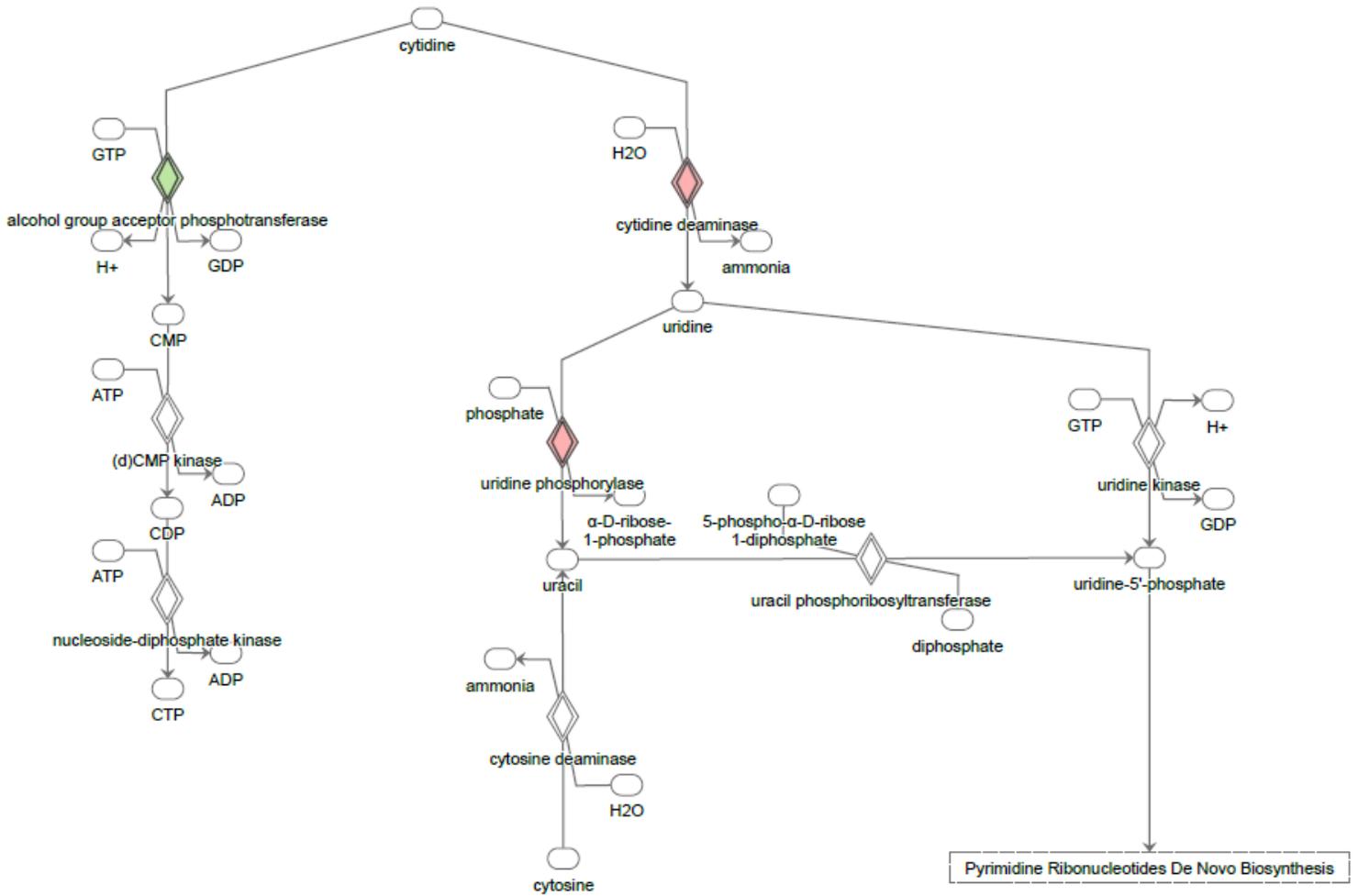


Figure 4

Salvage pathways of pyrimidine ribonucleotides. Genes marked in red are upregulated while those marked in green are downregulated between baseline and Day 2, in patients that did not achieve complete remission (nCR) after the first course of induction chemotherapy. Notably, increased expression of cytidine deaminase, responsible for the deamination of Ara-C into Ara-U, could be observed.

Supplementary Files

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