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***RRM1* and *RRM2* pharmacogenetics: association with phenotypes in HapMap cell lines and acute myeloid leukemia patients**

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Abstract

Background—Ribonucleotide reductase catalyzes an essential step in the cellular production of deoxyribonucleotide triphosphates and has been associated with clinical outcome in cancer patients receiving nucleoside analog-based chemotherapy.

Materials & methods—In the current study, we sequenced the genes *RRM1* and *RRM2* in genomic DNA from HapMap cell lines with European (Utah residents with northern and western European ancestry [CEU]; n = 90) or African (Yoruba people in Ibadan, Nigeria [YRI]; n = 90) ancestry.

Results—We identified 44 genetic variants including eight coding SNPs in *RRM1* and 15 SNPs including one coding SNP in *RRM2*. *RRM1* and *RRM2* mRNA expression levels were significantly correlated with each other in both CEU and YRI lymphoblast cell lines, and in leukemic blasts from acute myeloid leukemia (AML) patients (AML97, n = 89; AML02, n = 187). Additionally, *RRM1* expression was higher among patient features indicative of a high relapse hazard. We evaluated SNPs within the *RRM1* and *RRM2* genes in the HapMap lymphoblast cell lines from CEU and YRI panels for association with expression and cytarabine chemosensitivity. SNPs of potential significance were further evaluated in AML patients. *RRM1* SNPs rs1042919 (which occurs in linkage disequilibrium with multiple other SNPs) and promoter SNP rs1561876 were associated with intracellular 1-β-D-arabinofuranosyl-CTP levels, response after remission induction therapy, risk of relapse and overall survival in AML patients receiving cytarabine and cladribine.

Conclusion—These results suggest that SNPs within ribonucleotide reductase might be helpful predictive markers of response to nucleoside analogs and should be further validated in larger cohorts.

Keywords

cytarabine; HapMap; leukemia; nucleoside analogs; pharmacogenomics; ribonucleotide reductase

Ribonucleotide reductase (RR) is a key enzyme involved in the biosynthesis of deoxynucleotides. Since reduction in the levels of ribonucleotides is a rate-limiting step in DNA synthesis, inhibition of RR has been explored as a potential area for development of anticancer agents. The RR holoenzyme consists of the dimerized large and small subunits, *RRM1* and *RRM2*. RR regulates intracellular pools of deoxy-CTP (dCTP), which in turn, has been implicated in resistance to nucleoside analogs such as cytarabine (ara-C). Cell lines

and leukemic blasts with high levels of dCTP are resistant to ara-C [1–3]. Intracellular dCTP levels regulate ara-C metabolism at three levels: by feedback inhibition of DCK resulting in reduced activation of ara-C [4]; by allosteric activation of the inactivating enzyme CDA [3]; and by competing with the active metabolite 1- β -D-arabinofuranosyl-CTP (ara-CTP) for incorporation into DNA [5]. In addition to ara-C, higher intracellular levels of deoxynucleotides due to increased activity of ribonucleotide reductase can increase resistance to other nucleoside analogs such as cladribine using mechanisms mentioned above [6]. In fact biochemical modulation of ara-C by nucleoside analogs such as fludarabine and cladribine (that inhibits RR) has been shown to stimulate ara-CTP accumulation by inhibition of RR in leukemic cells from adult and pediatric patients [7–10].

RRM1 has also been implicated in suppression of cell migration and metastases, and its overexpression in Ras-transformed mouse fibroblasts decreases tumor development and metastases, suggesting a tumor suppressor role [11]. Overexpression of *RRM1* has also been shown to be protective against carcinogen-induced lung tumors in mice [11]. *RRM2* has been shown to be associated with tumorigenesis and drug resistance in preclinical studies [12]. Due to their key role in DNA synthesis/repair and in cell growth, *RRM1* and *RRM2* are targeted by several anti-cancer agents including nucleoside analogs (e.g., gemcitabine, cladribine and clofarabine) and hydroxyurea (reviewed by Shao *et al.* [13]). *RRM1* overexpression has been correlated with gemcitabine resistance in human pancreatic cancer [14], non-small-cell lung cancer cell lines and in mice with tumors [15,16].

In the present study, we first sought to identify genetic variation in *RRM1* and *RRM2* by resequencing the genomic DNA from HapMap European (Utah residents with northern and western European ancestry [CEU]) and African (Yoruba people in Ibadan, Nigeria [YRI]) ancestry panels. Then, we determined the association of SNPs (both from the HapMap database [101] and identified by resequencing) with mRNA expression levels and cytotoxicity to ara-C in HapMap cell lines. Finally, we investigated the clinical significance of *RRM1*, *RRM2* and *RRM2B* variants in pediatric acute myeloid leukemia (AML) patients who received ara-C along with cladribine treatment in the AML97 study and ara-C without cladribine in the AML02 study.

Materials & methods

Reagents

The Expand Hi Fidelity PCR system was obtained from Roche (IN, USA); the TOPOTA cloning kit and One Shot Mach1-T1 chemically competent *E. coli* cells were obtained from Invitrogen (CA, USA). Restriction enzymes, pGL3 basic vector and JM109 cells were obtained from Promega (WI, USA). RNeasy Minikit, QIA-prep Plasmid Miniprep kit and Plasmid Plus kit were from Qiagen Inc. All other reagents were of molecular biology grade.

HapMap cell lines

We used Epstein–Barr virus-transformed B-lymphoblastoid HapMap cell lines derived from 30 CEU trios (two parents and a child; n = 90, European descent) and 30 Yoruba trios (n = 90, African descent, referred to as YRI) to identify genetic variants in *RRM1* and *RRM2*. The purpose of using the same cell lines that have been used in the International HapMap project was to allow us to utilize the genotype data generated as part of the HapMap project. Genome-wide gene-expression data using Affymetrix (CA, USA) Exon array was used to extract the expression levels of *RRM1* and *RRM2* (GSE7761). Cells were grown in an RPMI 1640 medium supplemented with 2 mM L-glutamine (Lonza Walkersville, Inc., MD, USA) and 15% heat-inactivated serum at 37°C under 5% CO₂. DNA, RNA and cytoplasmic fractions were extracted from the cell lines using standard protocols. Genomic DNA was

used to discover novel genetic variants in the *RRM1* and *RRM2* genes. Ara-C cellular cytotoxicity was determined in a previous study using alamarBlue® (Biosource International, CA, USA). The area under the survival curve (AUC) was calculated using the trapezoidal rule and AUC values were log₂ transformed before statistical analysis as described earlier [17].

Identification of sequence variations in the *RRM1* & *RRM2* genes

All the coding exons including the flanking intronic sequences as well as 1.5 kb of the 5'-UTR of the genes *RRM1* and *RRM2* were PCR-amplified with primers designed using Primer Select module of Lasergene v 6.0 software (DNASTar) and synthesized at the University of Minnesota, Biomedical Genomics Center (MN, USA). The primer sequences were verified using UCSC BLAT [102] to exclude amplification of any non-specific DNA sequences. Information on individual primer sequences is provided in the Supplementary Material (see www.futuremedicine.com/doi/suppl/10.2217/pgs.13.131), PCR amplification, amplicon sequencing and analysis of sequences for identification of SNPs was performed as described previously [18].

Studies with primary leukemic cells

Bone marrow samples were obtained at diagnosis from pediatric AML patients enrolled in the St Jude AML97 [19] or St Jude AML02 [20] studies, after obtaining informed consent from their parents/guardians, with assent from the patients, as appropriate. The study was approved by the St Jude institutional review board.

The eligibility for the enrollment, treatment plan and clinical outcome of AML97 have been reported [19]. Because cladribine, an inhibitor of RR, had been shown to augment ara-CTP levels, patients were randomized to receive ara-C along with cladribine either as a daily short infusion or a continuous infusion. Bone marrow aspirates were obtained at diagnosis and after day 1 and 2 of ara-C treatment as described previously [21]. Leukemic cells were separated by Ficoll-Hypaque density-gradient centrifugation and intracellular ara-CTP levels were determined in samples obtained after day 1 and day 2 of ara-C treatment using HPLC as described earlier [21]. The patient population included 58% white patients, 22% black patients and 20% of patients were of other ethnic backgrounds.

Details of eligibility of enrollment, study design and clinical outcome for the AML02 protocol have been reported [20]. Briefly, patients were randomized to receive induction therapy containing either high-dose ara-C (3 g/m², given every 12 h on days 1, 3 and 5) or low-dose ara-C (100 mg/m²) given every 12 h on days 1–10 plus daunorubicin and etoposide as described earlier [20]. The patient population included 69.6% white patients, 18.7% black patients and 11.7% of patients were of other ethnic backgrounds. *In vitro* sensitivity of leukemic cells to ara-C was determined in patients enrolled in the AML02 study using the 4-day 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) cytotoxicity assay and LC₅₀ values were generated as part of a previously reported study [22,23]. *RRM1* and *RRM2* mRNA expression levels in diagnostic leukemic blasts were extracted from Affy U133A array data that was available from 207 patients (165 enrolled in AML02 and 42 in AML97).

Genomic DNA was extracted using DNA blood kit (Qiagen, CA, USA) and samples were evaluated for potentially functionally important *RRM1* (n = 14), *RRM2* (n = 9) and *RRM2B* (n = 3) SNPs representing major linkage disequilibrium (LD) groups in genomic DNA from AML97 (n = 89) and AML02 patients (n = 187) using Sequenom (CA, USA) based genotyping that uses MALDI-TOF-based chemistry at University of Minnesota, Biomedical Genomics Center.

Statistical analysis

The association of SNP genotype with *RRM1* and *RRM2* expression and AUC in HapMap cell lines was tested with an analysis of variance model that uses a Toeplitz correlation matrix [24] to account for the correlation of an individual's measurements with that of parents from HapMap trios. In AML patients, the Kruskal–Wallis test was used to compare expression levels and ara-C LC₅₀ values across groups of AML patients according to SNP genotype, clinical risk group or race. Spearman correlation coefficient was used to characterize the association of the number of minor alleles with minimal residual disease, mRNA expression, and ara-C LC₅₀ values. Event-free survival (EFS) was defined as the time elapsed from protocol enrollment to induction failure, relapse, withdrawal, secondary malignancy or failure with patients free of these events censored at last follow-up. Overall survival (OS) was defined as the time elapsed from protocol enrollment to death, with patients censored at last follow-up. Risk of relapse or resistant disease was defined as time elapsed from protocol enrollment to induction failure, relapse or competing events of withdrawal, secondary malignancy or death. The statistic of Jung, Owzar and George [25] was used to characterize the association of the number of minor alleles with EFS and OS. Cumulative incidence tests [26] or competing risks regression [27] was used to test association of SNPs with risk of relapse. All tests are two-sided and no multiple testing adjustments were performed in these exploratory analyses.

Results

SNPs identified in *RRM1* & *RRM2*

All the coding exons, flanking introns and the proximal promoter in CEU (30 trios with European ancestry, n = 90) and YRI (30 trios with African ancestry, n = 90) samples were sequenced. Figures 1 & 2 show the snapshots from UCSC genome browser [103] of regions sequenced in *RRM1* and *RRM2* along with key regions of functional importance (CpG site, 7× regulatory potential and conserved transcription factor binding sites). A total of 44 genetic variants in *RRM1* were identified by resequencing, while 15 SNPs were identified in *RRM2* (Figure 2 & Table 1). Among the 44 *RRM1* SNPs, ten were present in the promoter, eight in exons, 25 in intronic regions and one in the 3'-UTR (Table 1). Nineteen SNPs were found exclusively in the African ancestry (YRI panel) and nine exclusively in the European ancestry (CEU panel); 16 SNPs were common to both CEU and YRI groups. Fourteen SNPs overlapped with SNPs present in the HapMap database. Detailed results of the bioinformatic analysis are provided in Table 1. For *RRM2*, seven SNPs were present in the promoter, one in the exon and seven in intronic regions. Eleven SNPs were found exclusively in the African ancestry (YRI) group and one exclusively in the European ancestry (CEU) group; three SNPs were common to both CEU and YRI groups. Only one SNP (rs1130609) overlapped with SNPs present in the HapMap database (rel#28) [104].

Analysis of LD on SNPs identified in the current study and data from HapMap database [101] was performed using Haploview. We included 10 kb upstream and 5 kb downstream of the gene. For *RRM1*, the gene immediately upstream is *STIM1*, which occurs in a head-to-tail configuration with *RRM1*, with the 3' end of *STIM1* gene 1.6 kb from the 5' end of the *RRM1* gene. Based on the LD plot and results from tagger (Haploview) the strongly linked ($r^2 > 0.8$) *RRM1* SNPs in CEU and YRI were grouped into nine (CEU.1–CEU.9) and 17 (YRI.1–YRI.17) distinct groups as shown in Figure 1. For *RRM2* there were relatively few SNPs and hence few LD groups, specifically three in CEU (CEU.1: rs6759180, rs4668664 and rs718215; CEU.2 rs6741290 and rs4309551; CEU.3 rs7585544 and rs7582263) and one in YRI (rs6741290 and rs4309551 were in LD). SNPs that did not occur in linkage with other SNPs were termed as a 'singleton' (Figure 2).

Association of *RRM1* & *RRM2* SNPs with mRNA expression & cytotoxicity to nucleoside analogs in HapMap lymphoblast cell lines

mRNA expression in 87 CEU (representing 29 trios) and 90 YRI (30 trios with African ancestry) samples was obtained from the Gene Expression Omnibus database (GSE7761) [105]. *RRM1* and *RRM2* mRNA expression levels had wide interindividual variability and were significantly correlated with each other ($p = 0.0011$) in both CEU and YRI samples (Figure 3). We observed a significantly higher ($p < 0.0001$) *RRM2* mRNA expression in YRI compared with CEU samples. The cytotoxicity of ara-C for both CEU and YRI was previously reported [17]. Although expression levels of *RRM1* or *RRM2* were not significantly correlated with cellular sensitivity to ara-C in CEU and YRI, we did observe a trend ($p = 0.07$) for higher expression to be associated with greater AUC (higher AUC is associated with lower drug sensitivity) for cell survival in YRI (data not shown).

We analyzed the association of SNPs (identified by sequencing as well as from the HapMap database: 10 kb upstream and 5 kb downstream of the gene) with mRNA expression and cellular sensitivity to ara-C in HapMap lymphoblast cell lines (LCLs; Supplementary Table 1). None of the *RRM1* SNPs were associated with *RRM1* expression in CEU but rs1561876 was associated with ara-C cytotoxicity (GG vs GA vs AA, $p = 0.033$; Supplementary Table 1). In the YRI panel, promoter SNPs rs1465952 (YRI.2 occurs in LD with rs7945918) and rs1561786 (YRI.14 occurs in LD with rs7103860) were significantly associated with levels of expression of *RRM1* (Figure 4). Additionally, rs1465952 showed a trend and rs1561876 was significantly associated with ara-C cytotoxicity in the YRI panel (Supplementary Table 1 & Figure 4). Other SNPs upstream of *RRM1* that were significantly associated with ara-C cytotoxicity included rs2412344, rs11030907 and rs7929397 (Figure 4 & Supplementary Table 1). For *RRM2*, a coding SNP rs1130609 (S59A) was associated with ara-C cytotoxicity in CEU samples (Figure 5) and a promoter SNP rs7574663 was associated with its expression (Supplementary Table 1).

Relationship of *RRM1* & *RRM2* SNPs with treatment response in AML patients

A total of 11 SNPs within *RRM1* and seven in *RRM2* were genotyped in DNA samples from AML patients (Supplementary Table 2 provides list of SNPs and Supplementary Figure 1 provides LD groups for *RRM1* SNPs in AML patients). In addition, three SNPs in the *RRM2B* gene (rs7830150, rs17414857 and rs1265138) were also genotyped. The frequency of SNPs did not differ significantly between patients enrolled in the AML97 and AML02 studies ($p > 0.05$). SNPs occurring with the allele frequency of less than 10% were excluded, resulting in ten SNPs in *RRM1* (few of these SNPs were in linkage with others), four in *RRM2* and two in *RRM2B* for further analysis. After screening for LD, three additional SNPs in *RRM1* were excluded. As was observed for HapMap panels, the LD pattern was very different between white and black AML patients (Supplementary Figure 1).

RRM1 and *RRM2* mRNA expression data was extracted from Affymetrix U133A microarray data in diagnostic leukemia blasts from the St Jude AML02 study. As observed in HapMap LCLs, we observed significant correlation between *RRM1* and *RRM2* expression in leukemic blasts obtained at diagnosis from AML patients regardless of race ($p < 0.001$; Figure 6A & Supplementary Table 3). Higher *RRM1* expression was significantly associated with high-risk presenting features, such as -7, *FLT3*-ITD, t(6,9), megakaryoblastic leukemia, treatment-related AML or AML arising from myelodysplastic syndrome (Figure 6B). However, a similar significant trend was not observed for *RRM2* (data not shown). *RRM1* mRNA expression was significantly associated with the *RRM1* promoter SNPs rs11030918 ($p = 0.04$), rs12806698 ($p = 0.0004$) and rs1042927 ($p = 0.030$; Figure 7). The *RRM2* SNP rs1138729 was significantly associated with the mRNA expression of the *RRM2* gene ($p = 0.001$).

Patients enrolled in AML97 received the RR inhibitor cladribine in combination with ara-C. In a subset of patients within this cohort we had both *RRM1* and *RRM2* mRNA expression data (extracted from the Affymetrix U133A gene expression array) and intracellular ara-CTP levels in leukemic blasts determined at day 1 and 2 of therapy. We found an inverse correlation between *RRM1/2* mRNA expression and intracellular ara-CTP levels in leukemic blasts although it did not reach statistical significance (Supplementary Figure 2). The most interesting *RRM1* SNPs included rs1561876 (belonging to CEU.4) that occurs in LD with other SNPs (rs3794050 and rs7103860) in European ancestry (but only with rs7103860 in YRI). The variant allele of this SNP was associated with lower intracellular ara-CTP levels of leukemic blasts determined at day 1 of therapy ($p = 0.006$ among Caucasian patients and $p = 0.088$ in all patients); inferior response after the first course of remission induction therapy ($p = 0.0012$); poor EFS ($p = 0.009$); and greater risk of relapse ($p = 0.001$) in AML patients (Figure 8). Another *RRM1* SNP rs1042919, belonging to LD group CEU.1 (22 other SNPs occur in LD with this SNP, see Figure 1) was associated with lower intracellular ara-CTP levels in leukemic blasts on day 1 ($p = 0.006$; Figure 9A) and day 2 of therapy ($p < 0.05$), and poor outcome as shown by a worse EFS ($p = 0.0004$; Figure 9). In addition, *RRM1* rs2898950 and *RRM2B* SNP rs1265138 were associated with inferior response after induction therapy (Supplementary Figure 3). For *RRM2* SNPs, presence of variant allele for rs1130609 and rs5030743 was associated with poor EFS, although the number of patients with variant allele was small (Supplementary Figure 3).

For the AML02 cohort, we had *in vitro* ara-C cytotoxicity in a subset of patients ($n = 69$) determined by treating diagnostic leukemic cells with varying concentration of ara-C. None of the *RRM1*, *RRM2* and *RRM2B* SNPs were significantly associated with ara-C LC₅₀ in the combined cohort in analyses that ignored risk group. However, in analyses that stratified by risk group, *RRM1* SNP rs1042858 (occurs in LD with rs1042927) was associated with higher ara-C LC₅₀ as compared with patients with the AA genotype ($p = 0.03$; Supplementary Figure 4). *RRM1* promoter SNP rs1561876 (belonging to CEU.4 and YRI.13) showed a similar but nonsignificant association with prognosis as observed in AML97. No SNPs in *RRM2* and *RRM2B* were associated significantly with EFS, OS, minimal residual disease or RR in AML02 patients.

Discussion

In the present study we report results of sequencing of *RRM1* and *RRM2* genes and report on the evaluation of associations between mRNA expression and ara-C cytotoxicity in HapMap LCLs of European (CEU) and African ancestry (YRI). Overall SNPs within RR seem to be of lesser functional significance as few SNPs within the *RRM1* gene (predominantly in the YRI panel) were associated with mRNA expression and/or cellular sensitivity to ara-C in Hap-Map LCLs. We further evaluated association of *RRM1*, *RRM2* and *RRM2B* SNPs in two independent clinical cohorts of patients treated in the St Jude AML97 and St Jude AML02 studies.

The two most clinically interesting SNPs in *RRM1* included rs1042919 and rs1561876. The *RRM1* SNP rs1042919 represents the group CEU.1, occurs in LD with several other SNPs including rs1042858 (2464G>A; Ala744Ala), rs1042927 (2927A>C) and promoter SNP rs1662163 (−934), and was associated with lower intracellular ara-CTP levels in leukemic cells at day 1 (ara-C alone) and day 2 (ara-C in combination with cladribine). This SNP was also associated with poor survival and greater risk of relapse in patients treated with the AML97 cohort. Previous studies have shown that rs1042927 (also known as 2927) was associated with a significantly decreased survival ($p = 0.021$) in patients with malignant mesothelioma receiving gemcitabine-based chemotherapy [28]. For coding SNP rs1042858 (commonly known as 2464G>A) presence of the A allele has been previously reported to be

associated with greater chemosensitivity to gemcitabine [29], which is in agreement with our observation of the variant G allele being associated with poor clinical response as compared with the A allele.

The second most interesting SNP in *RRM1* is promoter SNP rs1561876 (present 2.5 kb upstream of exon 1 of *RRM1* and in 3'-UTR of neighboring gene *STIM1*), which occurs in LD with at least two SNPs within 10 kb upstream of *RRM1* in the CEU panel and with only one SNP in the YRI panel. rs1561876 C>T was associated lower ara-CTP levels at day 1 (post ara-C only) and day 2 (ara-C in combination with cladribine) poor response after induction 1 and EFS and greater risk of relapse in all and white patients within the St Jude AML97 cohort. However in Hap-Map LCLs it was associated with lower *RRM1* expression and greater ara-C chemosensitivity in the YRI panel (Figure 4). One of the reasons for this observation might be the fact that the LD structure of this gene is very different in European versus African ancestry. When screening for the region 20 kb upstream of *RRM1* we identified four additional SNPs in the upstream *STIM1* gene that were in LD with rs1561876 in CEU, but none in YRI. *STIM1* encodes a type 1 transmembrane protein that mediates Ca^{2+} influx after depletion of intracellular Ca^{2+} stores by gating of store-operated Ca^{2+} influx channels. *STIM1* is located 1.5 kb upstream of *RRM1* and is oriented in head to tail configuration with *RRM1*. Another point of consideration is the fact that several genes, including *RRM1* and *STIM1*, are located in the imprinted gene domain of chromosome 11p15.5. This region is an important tumor suppressor gene region with alterations implicated in Beckwith–Wiedemann syndrome, Wilms tumor, rhabdomyosarcoma, adrenocortical carcinoma, and lung, ovarian and breast cancer. We checked correlation of mRNA expression levels of *RRM1* and *STIM1* and observed significant correlation with both CEU and YRI HapMap LCLs ($p < 0.0001$ for both CEU and YRI), interestingly within AML patients the *RRM1* and *STIM1* expression levels were not correlated in diagnostic leukemic blasts ($p > 0.4$). It is possible that this might be one of the reasons for the observed differences in results between HapMap LCLs and AML patients.

Our results identified the *RRM1* SNP rs12806698 (-37A/C) to be associated with expression in AML02 samples, which was found to be consistent with the results from an earlier study [30]. This SNP has also been shown to be associated with response, toxicity sensitivity, survival and response to gemcitabine-based cancer chemotherapy in earlier studies [30–35]. Another SNP rs11030918 (-524C/T) was associated with mRNA expression in AML02 samples and has been related to the response rate in gemcitabine-based therapy [32,34]. It is possible that SNPs mentioned above or other SNPs that occur in LD with these alter mRNA/protein expression levels. The change in expression can influence cellular dCTP levels, which in turn can either, inhibit DCK or compete with ara-C for incorporation into DNA/RNA, both of which will result in inferior response to the chemotherapy. In addition to this it is also possible that altered RR levels/activity can interfere with its biological role in DNA synthesis/repair and cell growth thereby influencing the outcome.

For *RRM2*, the T allele of the SNP rs1130609 (S59A), was associated with greater ara-C sensitivity in HapMap samples (lower AUC) and diagnostic leukemic blasts (lower LC_{50}) and, accordingly, higher EFS and OS among AML97 patients. We also found one SNP in the gene *RRM2B*/p53R2 (rs1265138) to be associated with inferior response among AML97 patients (Supplementary Figure 3B). This is an interesting discovery since *RRM2B* expression has earlier been shown to be associated with better survival in patients with colorectal cancer [36].

For certain SNPs in *RRM1*/*RRM2*/*RRM2B*, we have not seen any association with mRNA expression with respective gene, but have observed significant association with ara-C cytotoxicity or clinical outcome. One of the explanations for this observation is that SNPs

(or SNPs in LD) might impact the secondary structure of RNA through post-transcriptional modifications. It is also possible that the presence of synonymous SNPs (e.g., *RRM1* rs1042927 occurs in LD with a synonymous SNP rs1042858 [2464G>A; Ala744Ala]) could influence translation kinetics by utilizing less abundant tRNA as has been previously shown for a synonymous SNP of P-gp [37].

Overall we observed more significant association of RR SNPs in the AML97 than in the AML02 cohort. One of the reasons for this observation may be that in the AML97 study patients received cladribine in combination with ara-C whereas in AML02 only ara-C-based therapy was used. Cladribine triphosphate is an inhibitor of RR and is expected to reduce cellular pools of deoxynucleotides thus lessening the feedback inhibition of deoxycytidine kinase. Clofarabine, a second generation nucleoside analog that also inhibits RRs and DNA polymerase is being investigated in combination with ara-C and our future work will further investigate the clinical significance of genetic variation in RRs identified in this study in specimens from patients receiving a combination of clofarabine and ara-C (multicenter AML08 study, clinicalTrials.gov identifier: NCT00703820). Our exploratory study of the association of *RRM1/RRM2* genotype with clinical outcomes among pediatric AML patients is one of the largest of its kind to date but nonetheless is limited by the small cohort of subjects with this rare disease. As such, our results should be considered as preliminary until confirmed in a separate cohort with a large sample size. We are currently attempting to obtain additional samples from other clinical trials to perform these confirmatory studies.

Conclusion & future perspective

In conclusion we evaluated SNPs within *RRM1* and *RRM2* for association with its expression and ara-C sensitivity to ara-C in HapMap LCLs from CEU and YRI panels. SNPs of potential significance were further evaluated in two independent patient cohorts. The most interesting SNPs were identified in the AML97 cohort, where patients received cladribine (RR inhibitor) in combination with ara-C. *RRM1* SNPs were associated with multiple clinical end points in AML patients and in most cases presence of a variant allele was associated with lower intracellular levels of ara-CTP in leukemic cells as well as inferior response and greater risk of relapse. The results of our study identified SNPs within RRs that were significantly associated with clinical end points in AML patients (especially in patients receiving RR inhibitor); however, within HapMap LCLs the results were not as impressive. Future work in larger cohorts is needed to further validate our results especially in patients receiving treatment with RR inhibitors. Our results also provide additional SNPs that should be tested in patients undergoing treatment with gemcitabine-containing chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Executive summary

Aim

- RRM1 and RRM2 are critical in regulating cellular deoxy-CTP pools and hence can influence drug sensitivity to nucleoside analogs. Thus, this study was designed to identify genetic variation in ribonucleotide reductase genes (*RRM1* and *RRM2*) and evaluate their relationship with response in acute myeloid leukemia (AML) patients.

Materials & methods

- HapMap cell lines from European and African ancestry were used for the discovery phase and specimens from AML patients from the St Jude AML97 and AML02 clinical trials were used to evaluate genetic variation in *RRM1* and *RRM2*.

Results

- We identified SNPs within *RRM1* and *RRM2* that were associated with either its expression and/or cytarabine chemosensitivity in HapMap cell lines.
- Evaluation in AML patients identified SNPs associated with intracellular levels of 1- β -D-arabinofuranosyl-CTP and clinical response in AML patients treated with a combination of cladribine and cytarabine.

Conclusion

- SNPs within *RRM1* and *RRM2* might be helpful predictive markers of response to nucleoside analogs and should be further validated in larger cohorts.
- Future *in vitro* functional studies are needed to understand the molecular mechanisms underlying the association of SNPs with the phenotype.

Figure 1. SNPs in *RRM1*
(A) Snapshot from the UCSC genome browser [NCBI/hg18 (2006)] for the *RRM1* locus. The regions sequenced in HapMap panels are shown by small boxes. (B) Linkage disequilibrium plot of *RRM1* gene in European (CEU) and African (YRI) ancestry samples generated in Haploview using genotype data from the present study and from HapMap for both CEU and YRI samples from 10 kb upstream to 5 kb downstream. (C) SNPs that are linked, $r^2 > 0.8$ (and picked by the tagger program), are categorized in the same groups. SNPs without rs numbers are indicated by number corresponding to Table 1. CEU: Utah residents with northern and western European ancestry; MAF: Minimum allele frequency; YRI: Yoruba people in Ibadan, Nigeria.

Figure 2. SNPs in *RRM2*

(A) Snapshot from the UCSC genome browser [NCBI/hg18 (2006)] for the *RRM2* locus.

The regions sequenced in HapMap panels are shown by small boxes. (B) Linkage disequilibrium plot of *RRM2* gene in European (CEU) and African (YRI) ancestry samples generated in Haploview using genotype data from the present study and from HapMap for both CEU and YRI samples 20 kb upstream to 5 kb downstream. (C) SNPs that are linked, $r^2 \geq 0.75$ in CEU and $r^2 \geq 0.8$ in YRI (and picked by the tagger program), are categorized in the same groups. SNPs located upstream of the translation start site (ATG) are denoted by an asterisk. SNPs without rs numbers are indicated by number corresponding to Table 1. CEU: Utah residents with northern and western European ancestry; MAF: Minimum allele frequency; YRI: Yoruba people in Ibadan, Nigeria.

Figure 3. Comparison of RRM1 and RRM2 mRNA expression in CEU and YRI cell lines
 RRM1 and RRM2 mRNA expression extracted from GSE7761 in Epstein-Barr virus-transformed lymphoblast cell lines derived from subjects with European (CEU) and African (YRI) ancestries.
 CEU: Utah residents with northern and western European ancestry; YRI: Yoruba people in Ibadan, Nigeria.

Figure 4. Association of SNPs with mRNA expression and cytarabine cytotoxicity in HapMap lymphoblast cell lines

Box plots for the association of representative *RRM1* SNPs with their expression and cytarabine area under the survival curve in YRI samples. Plots show medians as a line between boxes representing the first and third quartiles; the whiskers represent the range after excluding the outliers. The outliers are defined as data points that fall outside of the first and third quartiles by more than 1.5-times the interquartile range. Circles falling outside the whiskers represent outliers.

YRI: Yoruba people in Ibadan, Nigeria.

Figure 5. Association of *RRM2* SNP with cytarabine cytotoxicity in HapMap lymphoblast cell lines

Box plots for the association of representative *RRM2* SNPs with cytarabine area under the survival curve in CEU lymphoblast cell lines. Plots show medians as a line between boxes representing the first and third quartiles; the whiskers represent the range after excluding the outliers. The outliers are defined as data points that fall outside of the first and third quartiles by more than 1.5-times the interquartile range. Circles falling outside the whiskers represent outliers.

CEU: Utah residents with northern and western European ancestry.

Figure 6. Comparison of RRM1 and RRM2 mRNA expression in diagnostic leukemic blasts from acute myeloid leukemia patients

(A) RRM1 and RRM2 expression was extracted from Affymetrix U133A microarray data and correlation of mRNA expression levels between RRM1 and RRM2 was evaluated. (B) Box plot showing association of RRM1 expression with risk group. Plots show medians as a line between boxes representing the first and third quartiles; the whiskers represent the range after excluding the outliers. The outliers are defined as data points that fall outside of the first and third quartiles by more than 1.5-times the interquartile range. Circles falling outside the whiskers represent outliers.

Figure 7. Association of *RRM1* and *RRM2* SNPs with mRNA expression in diagnostic AML02 leukemic blasts

Box plots for the association of representative *RRM1* SNPs rs11030918, rs1042927 and rs12806698 with its expression and *RRM2* SNP rs1138729 with its mRNA expression levels in diagnostic leukemic blasts from acute myeloid leukemia patients. Plots show medians as a line between boxes representing the first and third quartiles; the whiskers represent the range after excluding the outliers. The outliers are defined as data points that fall outside of the first and third quartiles by more than 1.5-times the interquartile range. Circles falling outside the whiskers represent outliers.

Figure 8. Association of *RRM1* SNP rs1561876 with clinical outcome in AML97 patients
(A) Box plot representing association of rs1561876 with intracellular levels of ara-CTP (nmol/2 × 10⁷ cells) determined at day 1. Plots show medians as a line between boxes representing the first and third quartiles; the whiskers represent the range after excluding the outliers. The outliers are defined as data points that fall outside of the first and third quartiles by more than 1.5-times the interquartile range. The circle falling outside the whiskers represents an outlier. **(B)** Association of rs1561876 with response after induction 1 in acute myeloid leukemia (AML) patients. Percentages of patients in each category are indicated in parenthesis. **(C)** Survival curve for association of rs1561876 with event-free survival in all

AML patients. **(D)** Survival curve for association of rs1561876 with risk of relapse in AML patients.

Ara-CTP: 1- β -D-arabinofuranosyl-CTP; CR: Complete response; NR: No response; PR: Partial response.

Figure 9. Association of *RRM1* SNP rs1042919 with clinical outcome in Caucasian patients enrolled in AML97

(A) Box plot representing association of rs1042919 with intracellular levels of ara-CTP (nmol/ 2×10^7 cells) determined at day 1 in Caucasian acute myeloid leukemia (AML) patients. Plots show medians as a line between boxes representing the first and third quartiles; the whiskers represent the range after excluding the outliers. (B) Survival curve for association of rs1042919 with event-free survival in white AML patients. (C) Survival curve for association of rs1042919 with risk of relapse in white AML patients. Ara-CTP: 1- β -D-arabinofuranosyl-CTP.

Table 1
Genetic variants identified by sequencing *RRM1* and *RRM2* in HapMap lymphoblast cell lines (CEU and YRI).

rs no.	Change	Chromosomal position UCSC Hg18 (2006)	Position from translation start site (ATG) as +1	Location in the gene	Amino acid change	Bioinformatics (predictions)	HapMap/Novel	Allele frequency	
								CEU	YRI
RRM1									
1	rs1662162 T/C	4071840	-979	Promoter		Gain of V\$BCL6, V\$NOLF and V\$STAT sites; loss of V\$PERO site	HAPMAP_CEU, YRI	0.08	0.36
2	rs72555763 G/T	4071883	-936	Promoter			NOVEL_YRI		0.01
3	T/G	4072057	-762	Promoter		Gain of V\$HAND, V\$HEAT and V\$SORY sites; loss of V\$KLFS site	NOVEL_CEU	0.01	
4	rs11030918 T/C	4072063	-756	Promoter		Loss of V\$HEAT site	HAPMAP_CEU, YRI	0.35	0.16
5	rs1735068 T/G	4072234	-585	Promoter			HAPMAP_CEU, YRI	0.12	0.38
6	rs72555767 T/G	4072245	-574	Promoter		Gain of OSVTBP and V\$HEAT sites; loss of V\$ABDB, V\$BRN5, V\$BRNF, V\$CDXF, V\$FKHD, V\$RUSH and V\$SORY sites	NOVEL_YRI		0.02
7	rs72555753 C/A	4072411	-408	Promoter		Loss of V\$GLIF and V\$P53F sites	NOVEL_YRI		0.23
8	rs12806698 C/A	4072550	-269	Promoter		Gain of V\$DICE and V\$GLIF sites; loss of V\$CREB and V\$PAX5 sites	HAPMAP_CEU	0.29	
9	rs72555757 G/T	4072848	30	5'-UTR		Gain in V\$KLFS, V\$RXRF and V\$SPZ1 sites; loss of OSXCPE, V\$EGRF, V\$MAZF, V\$MZF1, V\$PAX3, V\$SPIF and V\$ZF02 sites	NOVEL_YRI		0.01
10	rs12801088 A/C	4072911	93	5'-UTR		Loss of V\$GLIF, V\$RREB and V\$SMAD sites	NOVEL_CEU, YRI	0.15	0.30
11	C/T	4079515	6697	Intron 1		Loss of 5SS, U2 site; loss of SF2/ASF, SF2/ASF (IgM-BRCA1) and SC35 sites	NOVEL_CEU		0.03
12	rs11030937 A/T	4079695	6877	Intron 1		Loss of branch site	HAPMAP_YRI		0.06
13	rs1348310 C/G	4079921	7103	Intron 2		Gain in SC35 sites	HAPMAP_CEU, YRI	0.09	0.20
14	rs232054 C/G	4080003	7185	Intron 2		Loss of branch site	HAPMAP_CEU, YRI	0.47	0.01
15	T/A	4083737	10919	Intron 2		Gain of SF2/ASF and SF2/ASF (IgM-BRCA1) sites	NOVEL_CEU	0.02	
16	T/C	4085278	12460	Exon 4	H108H		NOVEL_YRI		0.01
17	rs2228121 T/C	4085315	12497	Exon 4	L121L		NOVEL_YRI		0.06
18	rs725518 G/A	4085421	12603	Intron 4		Gain of SF2/ASF and SRP40 sites	HAPMAP_CEU, YRI	0.42	0.32

rs no.	Change	Chromosomal position UCSC Hg18 (2006)	Position from translation start site (ATG) as +1	Location in the gene	Amino acid change	Bioinformatics (predictions)	HapMap/Novel	Allele frequency	
								CEU	YRI
19	C/T	4085457	12639	Intron 4			NOVEL_YRI		0.01
20	C/T	4087599	14781	Intron 5		Loss of branch site	NOVEL_YRI		0.01
21	T/G	4087717	14899	Intron 5		Gain in branch site; loss of SRp40 and SRp55 sites	NOVEL_CEU	0.01	
22	G/C	4087733	14915	Intron 5			NOVEL_YRI		0.01
23	T/G	4087765	14947	Intron 5		Gain of donor splice site	NOVEL_YRI		0.06
24	G/A	4089611	16793	Intron 6		Gain in branch site; loss of SC35 and SRp40 sites	NOVEL_CEU	0.01	
25	C/A	4089893	17075	Intron 7		Loss of SRp55 site	HAPMAP_CEU, YRI	0.09	0.21
26	T/C	4097546	24728	Intron 8		Gain of SRp40 site	NOVEL_YRI		0.03
27	C/A	4097708	24890	Exon 9	R284R		HAPMAP_CEU	0.48	
28	A/G	4097801	24983	Intron 9		Gain of SRp40 site	NOVEL_YRI		0.01
29	T/C	4098371	25553	Intron 9			NOVEL_CEU	0.08	
30	T/C	4099663	26845	Intron 10		Gain of 5SS, U2 site; gain of SF2/ASF, SF2/ASF (IgM-BRCA1) and SRp40 sites; loss of SC35 site	HAPMAP_YRI		0.12
31	C/G	4099722	26904	Intron 10		Loss of SC35 site	HAPMAP_CEU, YRI	0.05	0.14
32	A/G	4099733	26915	Intron 10		Gain of SC35 site	HAPMAP_CEU, YRI	0.05	0.14
33	A/G	4099777	26959	Intron 10		Loss of branch site	NOVEL_YRI		0.06
34	A/T	4100018	27200	Exon 11	L370L		NOVEL_YRI		0.05
35	G/A	4106867	34049	Intron 14		Gain in branch site	NOVEL_YRI		0.13
36	A/C	4106955	34137	Exon 15	K590Q	Benign (0.004) [Polyphen1]; tolerated (0.35) [SIFT]	NOVEL_YRI		0.02
37	T/C	4109950	37132	Intron 15		Gain of SF2/ASF and SF2/ASF (IgM-BRCA1) sites	NOVEL_CEU	0.01	
38	C/T	4109983	37165	Intron 15			NOVEL_CEU, YRI	0.35	0.02
39	A/G	4109984	37166	Intron 15		Gain of SRp55 site	NOVEL_CEU, YRI	0.35	0.02
40	A/G	4110343	37525	Intron 16		Gain of branch site	NOVEL_CEU, YRI	0.08	0.19
41	C/T	4112962	40144	Exon 18	L692L		NOVEL_YRI		0.01
42	A/G	4116033	43215	Exon 19	T741T		NOVEL_CEU, YRI	0.47	0.09

rs no.	Change	Chromosomal position UCSC Hg18 (2006)	Position from translation start site (ATG) as +1	Location in the gene	Amino acid change	Bioinformatics (predictions)	HapMap/Novel	Allele frequency	
								CEU	YRI
43	rs1042858	G/A	4116042	Exon 19	A744A		NOVEL_CEU, YRI	0.08	0.24
44	rs1042919	T/A	4116340	3'-UTR			HAPMAP_CEU, YRI	0.09	0.19
RRM2									
1	rs72542776	C/T	10179706	Promoter		Gain in V\$BRAC and V\$MOKF sites; loss of V\$AP2F and V\$NR2F sites	NOVEL_YRI		0.08
2	rs72542778	C/T	10180005	Promoter		Gain in V\$RUSH, V\$SPIF and V\$ZF02 sites; loss of O\$XCPE, V\$EGRF and V\$OAZF sites	NOVEL_YRI		0.46
3	rs72542781	G/A	10180060	Promoter		Gain in V\$STAF, site; loss of O\$XCPE, V\$CTCF, V\$SETSF, V\$GLIF, V\$MAZF and V\$PLAG sites	NOVEL_YRI		0.03
4	rs72542782	G/A	10180118	Promoter		Gain in V\$HEAT, V\$PAX5 and V\$PAX6 sites; loss of V\$E2FF, V\$EGRF, V\$NRFI and V\$RXRF sites	NOVEL_YRI		0.10
5	rs72542788	C/G	10180316	5'-UTR		Loss of V\$HESF site	NOVEL_YRI		0.17
6	rs68002429	C/T	10180343	5'-UTR		Gain in V\$SMAD site; loss of V\$GREF and V\$NRSF sites	NOVEL_YRI		0.32
7	rs1130609	G/T	10180371	Exon 1	S59A		HAPMAP_CEU, YRI	0.26	0.03
8	rs72542793	A/C	10181292	Intron 3			NOVEL_YRI		0.01
9	rs5030743	C/G	10181325	Exon 4	S110S		NOVEL_YRI		0.13
10	rs6432065	C/T	10181508	Intron 4		Loss of SRp55 site	NOVEL_CEU	0.24	
11	rs6741290	C/T	10182160	Intron 4			NOVEL_CEU, YRI	0.41	0.38
12	rs72542796	A/G	10182185	Intron 4		Change in SRp55 site	NOVEL_YRI		0.04
13		C/T	10182249	Intron 4		Loss of SF2/ASF (Igm-BRCA1) and SC35 sites	NOVEL_YRI		0.03
14	rs77323676	C/T	10184314	Intron 5			NOVEL_YRI		0.12
15	rs61754180	T/C	10184662	Intron 6		Gain of SF2/ASF and SRp40 sites; gain in branchsite	NOVEL_CEU, YRI	0.03	0.01

CEU: Utah residents with northern and western European ancestry; YRI: Yoruba people in Ibadan, Nigeria.