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GENETIC BASIS FOR THE INCREASED EXPRESSION OF VACUOLAR H⁺ TRANSLOCATING ATPASE GENES UPON IMATINIB TREATMENT IN HUMAN LYMPHOBLASTOID CELLS

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Abstract

Purpose—The role of v-ATPases in cancer biology is being increasingly recognized. Yeast studies indicate that the tyrosine-kinase inhibitor imatinib may interact with the *v-ATPase* genes and alter the course of cancer progression. Data from humans in this regard is lacking.

Methods—We constructed 55 lymphoblastoid cell lines from pedigreed, cancer-free human subjects and treated them with IC20 concentration of imatinib mesylate. Using these cell lines, we: i) estimated the heritability and differential expression of 19 genes encoding several subunits of the v-ATPase protein in response to imatinib treatment; ii) estimated the genetic similarity among these genes and iii) conducted a high-density scan to find *cis*-regulating genetic variation associated with differential expression of these genes.

Results—We found that the imatinib response of the genes encoding v-ATPase subunits is significantly heritable and can be clustered to identify novel drug targets in imatinib therapy. Further, five of these genes were significantly *cis*-regulated and together represented nearly half-log fold change in response to imatinib ($p = 0.0107$) that was homogenous ($p = 0.2598$).

Conclusions—Our results proffer support to the growing view that personalized regimens using proton pump inhibitors or v-ATPase inhibitors may improve outcomes of imatinib therapy in various cancers.

Keywords

chronic myeloid leukemia; metastasis; imatinib; microarray

INTRODUCTION

The first generation tyrosine-kinase inhibitor, imatinib, has become a mainstay in the treatment of chronic myeloid leukemia (CML) and has resulted in dramatic improvements in disease outcome.^{1–3} Additionally, the use of imatinib now extends beyond CML to

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gastrointestinal stromal tumors, Kaposi's sarcoma and glioblastomas.⁴⁻⁶ However, the widespread use of imatinib has led to a growing recognition of drug resistance,^{2, 3} and the necessity for continuous treatment gives rise to issues of toxicity in a substantial proportion of subjects.^{3, 7} Imatinib toxicity can lead to a spectrum of symptoms including diarrhea, vomiting, weight gain, edema, ascites, pleural effusion, pulmonary edema, fatigue, depression, desquamation, gastrointestinal hemorrhage, hyperbilirubinemia and somatic pain,⁷ as well as unexpected and unexplained cardiotoxicity.⁷⁻⁹ While genetic influences have been postulated to contribute to imatinib toxicity, the molecular pathways involved are currently not well-understood.

In this regard, there is now burgeoning indication that the vacuolar H⁺ translocating ATPase (v-ATPase) protein may be an important player in the genomic cascade of the effects of imatinib toxicity. For example, a recent genome-wide study¹⁰ in yeast demonstrated that a total of 51 genes are required for imatinib resistance, including a category of genes related to vacuolar pH homeostasis. Further, we recently implicated vacuole/lysosome related genes as potential targets of imatinib treatment in cell-based gene expression assays.¹¹ v-ATPase is a highly evolutionarily conserved protein localized in the vacuolar membrane as well as the plasma membrane (Figure 1A).^{12, 13} It has two domains (V0, buried inside the membrane and V1, protruding peripherally) and 13 subunits (a through e for V0 domain and A through H for the V1 domain), that are together encoded by several genes located on various chromosomes. The v-ATPase protein serves several important functions, of which the two most important ones are proton pumping (mainly through the V0 domain) and ATP hydrolysis (V1 domain),¹⁴ which both contribute to pH maintenance. Given that tumor cells thrive in an acidic microenvironment,¹⁵ potential alteration of v-ATPase expression by imatinib treatment may have important implications for tumorigenicity and metastasis.

In this vein, we conducted toxicity and association studies in 55 lymphoblastoid cell lines derived from cancer-free subjects with two objectives. First, we set out to investigate whether there is a significant differential expression of the *v-ATPase* genes in response to imatinib treatment *in vitro* and whether this differential expression can be partially explained genetically. Second, we examined whether imatinib-induced differences in *v-ATPase* gene expression is *cis*-regulated. Here, we report our findings of imatinib-altered *v-ATPase* gene expression and its *cis*-regulation in human cell lines.

METHODS

Cell lines and viability studies

We used Epstein-Barr virus-immortalized lymphoblastoid cell lines derived from 55 pedigreed Mexican American individuals in 17 families participating in the San Antonio Family Heart Study.¹⁶ The kinships pairings revealed siblings (72), third degree relations (15) and an identical sib pair. The mean age of study subjects at the time of lymphocyte collection was 43.23 ($\pm$ 13.59) years. Cells were maintained, as previously described,¹¹ in complete RPMI 1640 medium (Life Technologies, Grand Island, NY) at 37°C and 5% CO₂. Imatinib mesylate (LKT Laboratories, St Paul, MN) was solubilized in water at 10mM concentration, and stored at -20°C. Stock solutions were further diluted in water and then media for all assays. We had previously undertaken a study to investigate cytotoxicity of imatinib mesylate by generating dose-response curves to determine the heritability of various inhibitory concentrations (IC) and to assess gene expression changes.¹¹ All 55 cell lines were treated with a concentration of imatinib mesylate needed to inhibit 20% of the cells (IC₂₀) for a period of four days (96 hours); this concentration allowed us to maintain the viability of the majority of cells, whilst allowing us to examine gene expression changes that might be pertinent to imatinib cytotoxicity (for more detailed methodology, see ¹⁷).

Gene Expression Assays

Each of the 55 selected cell lines was treated with an IC20 dose of imatinib (calculated independently for each cell line), or was left untreated, and RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA concentration was determined using the NanoDrop ND-1000 (ThermoScientific, Wilmington, DE) and integrity assessed using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Anti-sense RNA (aRNA) was synthesized, amplified and purified from 500ng total RNA following manufacturer's guidelines for the Ambion MessageAmp II-Biotin Enhanced Single Round aRNA Amplification kit (Life Technologies). A total of 1.5µg aRNA was hybridized to Illumina Human WG-6 v3 BeadChips according to manufacturer's instructions and scanned using the Illumina® BeadArray™ 500GX Reader with Illumina® BeadScan image data acquisition software (version 2.3.0.13). Quality control procedures included a total RNA control sample and assessment of control summary reports, hybridization signal, background signal and the background-to-noise ratio for all samples analyzed. Illumina® BeadStudio software (version 1.5.0.34) was used for preliminary data analysis, with a standard background subtraction, to generate an output file for statistical analysis.

Vacuolar ATPase genes

The Illumina Human WG-6 Bead-Chip contained 29 probe sets designed to detect 19 genes encoding various subunits of both the V0 and V1 domains of the vacuolar ATPase protein. The genomic locations and the related probe sets of these genes are shown in Table 1. If a gene had more than one related probe sets then we used the average of the related probe sets as the expression for that gene.

High density single nucleotide polymorphism genotyping

Individuals were genotyped using a combination of Illumina BeadChips (HumanHap550v3, HumanExon510Sv1, Human1Mv1 and Human1M-Duov3), following the Illumina Infinium protocol (Illumina, San Diego, CA). Quality control excluded single nucleotide polymorphisms (SNPs) based on low call rates, monomorphic genotypes, <10 individuals with the minor allele and Hardy-Weinberg Equilibrium test statistics with $p < 10^{-4}$ (calculated by taking pedigree relationships into account). Thus, we had a total of 944,565 SNPs available for final analysis. SNP genotypes were checked for Mendelian consistency using SimWalk2. MERLIN was used to impute missing genotypes conditional on relatives' genotypes, with a weighted average of possible genotypes being used when an individual's genotype could not be inferred with certainty.

Statistical analysis

We expressed the response of gene expression to imatinib treatment as log fold-change. The results were normalized using quantile normalization as described elsewhere.¹⁸ For each probe (or average of probes for genes detected by multiple probes), we tested whether the difference in gene expression was significantly different from zero. Since the dataset originated from related individuals, we corrected for the within-family correlations and potential kinship effects. We tested the significance of response to imatinib using the following sporadic model:

$$\log FC = m + \beta\alpha + e_i \quad (1)$$

where FC is fold change in response to a imatinib, m is average fold change attributable to the drug treatment after accounting for kinship and covariates, β is the regression coefficient vector corresponding to the covariate matrix α and e_i is the measurement error. The covariates used in all models were age, sex, age $\times$ sex interaction, age² and age² $\times$ sex

interaction. The statistical significance of m was tested by constraining it to zero and estimating $\chi^2(1)$ as twice the difference between the log-likelihood from un-constrained and constrained models. To estimate the heritability of gene response to imatinib, we used the following polygenic model:

$$\log FC = m + \beta\alpha + p + e_i \quad (2)$$

where p is the polygenic effect and all other covariates are the same as those in equation (1). The statistical significance of heritability was tested by estimating $\chi^2(1)$ as twice the difference between the log-likelihoods for equations (1) and (2).

For clustering of the 19 genes included in this study, we used multidimensional scaling based on a dissimilarity matrix. For constructing this matrix, we first conducted bivariate analyses and estimated the genetic correlation coefficient (ρ_g) between each pair of the genes based on their response to imatinib treatment. Dissimilarity was then determined as $(1 - \rho_g^2)$ and the resulting dissimilarity matrix was input to multidimensional scaling. Lastly, we determined the *cis*-regulation of the *v-ATPase* genes by testing for associations with a total of 93 SNPs that were within 100 kb flanking region around studied genes using the measured genotype approach.

All genetic analyses were conducted using the Sequential Oligogenic Linkage Analysis Routines (SOLAR) software package¹⁹ (Version 6.3.7, Texas Biomedical Research Institute, San Antonio, TX) using the inverse normal transformation of each trait. Statistical analyses were done using the Stata software package (Version 12.0, Stata Corp, College Station, TX). All statistical tests were conducted for global type I error rates of 0.05.

RESULTS

We previously found that the heritability of imatinib IC20 values was 0.60 ($p=5.8 \times 10^{-4}$) and had identified several genes showing differential expression in response to imatinib treatment.¹¹ In particular, k-means clustering and DAVID functional annotation showed expression changes in genes related to kinase binding and vacuole-related functions following treatment of cell lines with an IC20 dose of imatinib.¹¹ We therefore further investigated a subset of these genes, the *v-ATPases*, which encode the vacuolar H⁺ translocating ATPase protein. We estimated the imatinib response (that is, log fold-change in gene expression upon imatinib treatment) for the expression of each of the 19 genes included in this study. Gene expression of ten (53%) of these genes (*ATP6V0D1*, *ATP6V0E2*, *ATP6V1A*, *ATP6V1B1*, *ATP6V1B2*, *ATP6V1D*, *ATP6V1E2*, *ATP6V1F*, *ATP6V1G1* and *ATP6V1G3*) showed a statistically significant heritability that exceeded 0.60 (Table 1). Second, we investigated gene expression changes associated with response to imatinib treatment. We found that ten genes (53%) exhibited a near or complete statistical significance and were up-regulated upon imatinib treatment. These genes were: *ATP6V0C*, *ATP6V0D2*, *ATP6V1A*, *ATP6V1B1*, *ATP6V1B2*, *ATP6V1D*, *ATP6V1E1*, *ATP6V1E2*, *ATP6V1F* and *ATP6V1G1*. Of these, four genes (*ATP6V1B1*, *ATP6V1B2*, *ATP6V1D* and *ATP6V1F*) had an association p-value <0.05.

Considering the observed genetic basis to the differential expression of these 19 genes in response to imatinib, we then aimed to cluster them based on their genetic dissimilarity (Figure 1B). We observed that *ATP6V0B* was uniquely placed away from all other genes which could be clustered into four groups. The largest of these groups was formed by *ATP6V0D1*, *ATP6V0D2*, *ATP6V0E1*, *ATP6V1A*, *ATP6V1B1*, *ATP6V1B2*, *ATP6V1D*, *ATP6V1F* and *ATP6V1G1*. Interestingly, when compared with the *v-ATPase* protein

structure, most of these genes encoded the stalk and tri/hexameric regions that partake in ATP hydrolysis.

Lastly, we examined whether the imatinib response of these genes is *cis*-regulated. For this, we assessed the 100,000 base flanking areas around these genes to identify SNPs that showed the most significant association with gene expression changes in response to imatinib. After accounting for the number of SNPs examined for each gene, we found eight genes that demonstrated statistically significant *cis*-association (Table 1, column titled “*Cis*-acting SNPs”). Of these, five belong to the aforementioned cluster of genes encoding the stalk and tri/hexameric regions of v-ATPase. We therefore proceeded to estimate the average response of these five genes (*ATP6V0D2*, *ATP6V1A*, *ATP6V1B1*, *ATP6V1B2* and *ATP6V1F*) to imatinib with more intensive multivariate trait analyses in SOLAR. We first ran a general model (with the mean expression of the five genes permitted to be estimated in a sporadic model), then ran a null model (all five means constrained to zero) and an alternative model (all five means constrained to be equal). Average response to imatinib for the five genes was estimated using the alternative model whilst the between-gene heterogeneity was estimated by comparing the null and general models. Using this approach, we found that the average differential expression of the five genes in response to imatinib was 0.49 (95% confidence interval 0.12 – 0.87, $p = 0.0107$) while the between-gene heterogeneity was not significant (Q statistic 5.28, $df = 4$, $p = 0.2598$).

DISCUSSION

Our results demonstrate that a high dose of imatinib may up-regulate key genes encoding various subunits of the v-ATPase enzyme; and that this up-regulation is a heritable trait which is *cis*-regulated. These results are of immediate relevance to imatinib use, especially in CML. The v-ATPases are strategically located in a cell so that they drive the H^+ ions away from the cytosol¹⁵ – either towards the inside of vacuoles or out of the plasma membrane. Their action therefore acidifies the vacuoles and the extracellular milieu. It is this latter consequence that is of importance in cancer biology since tumor cells prosper in an acidic microenvironment.¹³ Our results suggest that higher doses of imatinib may lead to an acidic microenvironment, via up-regulation of v-ATPase. Such a favorable environment may foster metastasis or imatinib resistance.²⁰ This supports the view that supplemental therapy with proton pump inhibitors or specifically, v-ATPase inhibitors may be beneficial in arresting cancer progression. Even more specifically, our results indicate that genetic variation within the stalk and tri/hexameric regions of the v-ATPase molecule may be partially driving the response to imatinib. The fact that up-regulation of *v-ATPase* gene expression is partially genetically determined also has important translational implications. These observations provide leads into the possibility of personalizing cancer treatments based on the genetic predilections and variations. Direct functional evidence of the role of the *cis*-regulating SNPs identified here has not yet been investigated and our findings give possible leads for further research in the treatment of CML with imatinib.

We had previously found a high degree of heritability for imatinib cytotoxicity (0.60 at an IC₂₀ dose), and here we show that following imatinib treatment, gene expression changes of several *v-ATPase* genes are also heritable (0.62–0.99), emphasizing the importance of genetics in an individual's response to imatinib. Previously, Watters and colleagues had found that 5-fluorouracil cytotoxicity was also heritable (0.26–0.65, dependent on dose),¹⁷ showing similar heritabilities to those reported for imatinib mesylate in our studies (0.60–0.73, dependent on dose).¹¹ More recently, Peters et al²¹ have described the heritability of cytotoxicity in response to 29 anti-cancer drugs that have been approved by the United States Food and Drug Administration. In that study, the heritability estimates derived from

125 cell lines varied from 0.06 to 0.64 with only two drugs (epirubicin and temozolomide) showing heritability of cytotoxicity comparable to that found for imatinib in this study.

Although we provide here, evidence for a role of the *v-ATPase* genes in imatinib cytotoxicity, we must also acknowledge the limitations of this study. This study was conducted in lymphoblastoid cell lines, using a dose of imatinib higher than what is required in clinical practice, but sufficient to inhibit cell growth (20% of cells) of non-cancerous cells. Also, we have examined only a relatively small number of cell lines which were all derived from a single ethnic population, Mexican Americans. Lastly, our inferences are based on gene expression of *v-ATPase*, rather than protein expression. Given the complexity implicit in the orchestration of the v-ATPase protein by the cellular machinery,²² it is possible that some discordance between the gene and protein expression may be operative. A study in yeast has shown that removal of individual *v-ATPase* genes is sufficient to render yeast susceptible to imatinib treatment,¹⁰ and as such, analysis of gene expression may still be important in understanding the physiology of imatinib treatment. Further, many studies have outlined the utility of studying gene expression to aid in diagnosis, risk stratification and response to drugs, including imatinib mesylate.^{23, 24} Finally, as we have shown in this study, the coordinated examination of structural genetic data (polymorphisms) and gene expression, allows identification of *cis*-regulatory variants that may be pertinent to understanding the disease process. However, future studies, aimed at establishing the relationship between gene and protein expression, are necessary to fully comprehend the role of the v-ATPase protein in response to imatinib treatment.

This is the first study in human cells showing an increase in the expression of *v-ATPase* genes upon imatinib treatment. Such an increase has also been shown in response to other anti-cancer drugs like cisplatin.²⁵ Our results provide potentially new directions for novel drug targets designed to ameliorate or prevent the growing prevalence of imatinib resistance and toxicity.

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Abbreviations used

ATP	adenosine triphosphate
CML	chronic myeloid leukemia
SNP	single nucleotide polymorphism
SOLAR	Sequential Oligogenic Linkage Analysis Routines

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Figure 1. Subunits of the human v-ATPase protein and the similarity of the genes encoding these subunits

(A) Conceptual schematic of the structure of the v-ATPase protein. V0 is the transmembrane domain while the V1 domain hangs outside the membrane. Lower case letters are used to show the subunits of the V0 domain (a through e) and upper case letters are used for the subunits of the V1 domain (A through H). The subunits A, B, E and G are trimeric and the stalk is formed by the d, D and F subunits. **(B)** Results from multidimensional scaling of the genetic correlations between pairs of the genes encoding subunits of the v-ATPase protein. The inset shows the location of the subunits encoded by the cluster of genes color-coded as pink. Colored subunits are encoded by the clustered genes.

Table 1

Heritability and mean response to imatinib treatment of *v-ATPase* genes.

Gene	Chr	Illumina probes used	Heritability		Differential gene expression			Cis-acting SNPs			
			h ² r	p	FC	p	#SNPs	Best SNP	Bon-p	Distance	
ATP6V0B	1	ILMN_1721391, ILMN_2353642	0.00	0.5000	0.09	0.3841	5	rs730916	0.6492	9788	
ATP6V0C	16	ILMN_1653056, ILMN_1773849, ILMN_1789005	0.00	0.5000	0.39	0.0596	3	rs11642783	0.1322	81653	
ATP6V0D1	16	ILMN_1795826	0.99	0.0230	0.29	0.1263	4	rs1548912	0.4422	-11307	
ATP6V0D2	8	ILMN_1799889	0.00	0.5000	0.40	0.0580	6	rs7816915	0.0430	64095	
ATP6V0E1	5	ILMN_1715635, ILMN_2071937	0.23	0.1520	0.12	0.3156	5	rs17069810	0.0039	-97264	
ATP6V0E2	7	ILMN_1785095	0.62	0.0142	0.21	0.2134	2	rs855913	0.6852	-80965	
ATP6V1A	3	ILMN_1711516	0.76	0.0058	0.39	0.0715	6	rs4682144	0.0157	76363	
ATP6V1B1	2	ILMN_1812073	0.88	0.0020	0.56	0.0163	3	rs3771389	0.0184	-39295	
ATP6V1B2	8	ILMN_1787705	0.75	0.0091	0.46	0.0473	8	rs6994455	0.0060	43915	
ATP6V1C1	8	ILMN_1659801, ILMN_1663257	0.00	0.5000	-0.18	0.2359	5	rs7016814	0.3189	-16025	
ATP6V1C2	2	ILMN_1660729, ILMN_2389995	0.00	0.5000	0.29	0.1222	6	rs9287724	0.2628	63638	
ATP6V1D	14	ILMN_1797310	0.80	0.0092	0.51	0.0307	3	rs7153102	0.0577	89063	
ATP6V1E1	22	ILMN_2339779, ILMN_1798485	0.00	0.5000	0.38	0.0661	0	-	-	-	
ATP6V1E2	2	ILMN_1810235	0.93	0.0002	0.38	0.0752	6	rs7591561	0.1363	44591	
ATP6V1F	7	ILMN_2099783	0.60	0.0344	0.57	0.0167	8	rs11975930	0.0191	37245	
ATP6V1G1	9	ILMN_1784523	0.86	0.0026	0.36	0.0936	3	rs9314737	0.1356	-59818	
ATP6V1G2	6	ILMN_1654541, ILMN_1674778	0.26	0.1086	0.14	0.2986	5	rs2844477	0.0413	66198	
ATP6V1G3	1	ILMN_2371001, ILMN_1726273	0.64	0.0237	0.40	0.2166	4	rs12759907	0.0195	66409	
ATP6V1H	8	ILMN_1689473, ILMN_2403730	0.18	0.2525	-0.03	0.4580	11	rs182914	0.2338	576	

Chr, chromosome; Start, base location of start of gene; h^2_r , heritability; FC, mean log-fold change in gene expression in response to imatinib treatment; SNP, single nucleotide polymorphism; #, number of SNPs fulfilling cis-regulating criteria and showing significant association with differential gene expression; best, SNP showing the most significant cis-association; Bon-p, significance value after correction for the number of SNPs using the Bonferroni procedure; Distance, genomic distance (bases) between the transcription start site and its most significant cis-regulating SNP