Exhibit 3

REVIEW

Gene variants in noncoding regions and their possible consequences

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Human biodiversity or individual traits are not well explained by exonic mutations of all 20,000 known human genes. Accumulating evidence has demonstrated that not all noncoding regions are junk DNA sequences, and that some functionally important noncoding variants contribute significantly to altered gene expression, qualitatively or quantitatively. Thus, functional profiling or clinical relevance of noncoding variations should not be underestimated or ignored. To validate these concepts, some important examples are discussed further in this short review.

Keywords: 3′-untranslated region, 5′-untranslated region, intron, mutation, noncoding region, pharmacogenetics, pharmacogenomics, polymorphism, promoter, regulation, transcription, variation

Over the past few decades, in particular after the completion of the Human Genome Project, there has been an explosion of information regarding the study of genomic structure–function relationships. This advances better understanding of human biodiversity or individual variability in response to diverse environmental stimuli and therapeutic treatments. According to a recent estimation, over 99.9% of human genome sequences are thought to be similar or identical, whereas less than 0.1% may vary, leading to individual traits [1]. These tiny differences in human genetic makeup include single nucleotide polymorphisms (SNPs), insertions, deletions (nucleotides or whole gene), inversions, rearrangements, and copy-number polymorphisms, such as variable number of tandem repeats (VNTRs), and duplications and multiplications of certain genes. A large number of studies have demonstrated that some (but not all) DNA variants present in the gene coding regions can cause amino acid substitutions, frame shifts, or truncated proteins, resulting in abnormal protein structure and function. In contrast, the noncoding regions, which constitute promoter regions, 5′-untranslated regions (5′-UTR), intronic regions, 3′-untranslated regions (3′-UTR), and intergenic regions (large regions between genes) [2], can often function as regulatory sequences that affect gene splicing, transcription and translation. Thus, some noncoding polymorphisms can alter gene expression. In this review, noncoding DNA variations and their possible functional significance are discussed.

Variation in the 5′-untranslated regions

The 5′-UTR is defined as the untranslated DNA sequences upstream of the translation start codon (ATG) of a gene. Although they are unable to be translated into amino acids, they act as an important regulatory sequence, affecting their gene expression levels through indirect or direct mechanisms.

Variants that are not localized at the binding sites of transcription factors or nuclear receptors

A well-characterized example is that of noncoding polymorphisms in the vitamin K epoxide reductase complex (*VKORC*)*1* gene, which can affect transcription and alter warfarin dose requirements [3]. In this study, ten common SNPs were identified within the promoter region of the *VKORC1* gene and five major haplotypes inferred, and *VKORC1* mRNA levels varied according to the haplotype combination. Furthermore, haplotypes 1 and 2 were collectively designated as the haplotype group A, as both were shown to be associated with a low-dose requirement of warfarin, whereas haplotypes 7–9 (or haplotype group B) were responsible for a high-dose requirement. In addition, Asian patients [3], in particular those of Chinese origin, have a high frequency of the haplotype group A that predicts the low-warfarin-dose phenotype, whereas African–Americans have a relatively high frequency of the haplotype group B, compared with white patients. Interestingly, promoter polymorphisms in the human *VKORC1* gene (encoding the target of warfarin) have greater influence on warfarin dose requirements than exonic polymorphisms in the human cytochrome P450 (*CYP*)*2C9* gene that encodes a major drug-metabolizing enzyme of *S*-warfarin [4].

Another example is the variation in the gene encoding α_{2B}-adrenergic receptor (ADRA2B), and its relationship to vascular response *in vivo* [5]. ADRA2B plays an important role in vasoconstriction and blood pressure regulation, but little is known about its pharmacogenomics.

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In a retrospective cohort study, DNA samples were obtained from a cohort group of 68 subjects who had vascular phenotype data, and the sequencing of this gene was performed. In total, 24 variants (50% in the promoter region, 21% in the coding region, and 29% in the 3′-UTR) were identified and four major haplotypes inferred. Compared with the noncarriers, subjects bearing haplotype 4, that is characterized by two SNPs in the 5′-UTR and one in the 3′-UTR, occurred only among African–Americans at the haplotype frequency of 18% (7/38), relating to a significantly reduced maximal response to increasing doses of the highly selective α_2-adrenergic receptor agonist, dexmedetomidine. This case demonstrates that such a particular haplotype may cause reduced receptor expression, consequently resulting in reduced maximal effects.

Recently, the authors (PY) observed that a common cardiac sodium channel promoter haplotype (a set of six sodium channel voltage-gated, type V α [*SCN5A*] promoter polymorphisms, designated HapB relative to its wild-type HapA) occurred at an allele frequency of 22% in East Asian subjects and was absent in black and white subjects [6]. Compared with the wild-type, this variant haplotype is associated with reduced transcriptional activity (as assessed by luciferase expression) in transient transfection assays and slowed cardiac conduction velocity (as assessed by PR and QRS durations) in both a Japanese control population and patients with Brugada syndrome [6]. At baseline, control subjects homozygous for the HapB had longer QRS durations than patients homozygous for the wild-type. When challenged with sodium channel blockers, the patients homozygous for the HapB had the longest QRS durations [6].

The three examples noted previously suggest that screening for functionally significant variants in the noncoding regions should receive similar priority for their potential to impact expression or function of the underlying gene relative to polymorphisms in its coding regions.

Variants that occur in the binding sites of transcription factors or nuclear receptors

Recent studies have shown that constitutive, induced, and repressed expression of genes important to the drug metabolism and transport are largely under the transcriptional control of certain nuclear receptor family members, of which constitutive androstane receptor (CAR), pregnane X receptor (PXR), vitamin D receptor (VDR), hepatic nuclear factor (HNF) family members HNF1α and HNF4α, and small heterodimer partner-1 (SHP-1) have been identified as functionally important transcription factors of drug disposition genes [7]. The DNA binding domain of each nuclear receptor protein binds to its corresponding nuclear receptor binding element present in a specific recognition site, triggering subsequently altered transcriptional activity. For example, CAR, PXR, HNF4α, and possibly, HNF1α binding sites exist within -3 kb 5′-upstream region of human *CYP2C9* gene, and thus these coactivators (such as HNF4α or HNF1α) are shown to upregulate PXR or CAR-mediated *CYP2C9* transcription, alone and in combination [8–12]. In contrast, SHP-1, generally as a corepressor, suppresses the gene transcription [12].

As for the significance of their variants, one striking observation is that 1,25-dihydroxy-vitamin D3 can induce gene expression of *CYP3A4* and not of *CYP3A7*, presumably because VDR cannot bind effectively to the *CYP3A7* promoter binding site due to mutations in its proximal ER6 motif [13]. The other example is that multiple SNPs in the *HNF4α* promoter have been associated with susceptibility to type 2 diabetes [14]. In addition, a most frequently cited example is that 5-lipooxygenase (*ALOX5*) gene promoter polymorphisms confer a reduced response to targeted treatment for asthma [15]. For this genotype–phenotype correlation study, the core promoter of the gene *ALOX5* harbors 3–6 tandem repeats of the Sp1 binding domain (GGGCGG) among individuals. When the most common allele (VNTR = 5) is designated as the wild-type allele, the other alleles (VNTR = 3,4,6) are named as mutant alleles (because they also have been shown to have reduced promoter–reporter activity in human cells [16]). Based on these observations, patients carrying different promoter sequence copy number variants may exhibit different levels of the gene transcription and translation, and thus affect clinical response to the drug targeting ALOX5. As expected, patients homozygous for the mutant alleles failed to response to ALOX5 inhibition [15].

Variants in the upstream open reading frame

Upstream open reading frame (uORF) exists within the 5′-UTR of some, but not all, genes. Previous studies have demonstrated that the presence or overexpression of the uORF can suppress translation of its downstream major

ORFs [17–19]. The underlying mechanisms may depend on the configuration of the uORF relative to the downstream major ORFs and their primary sequence. A common uORF polymorphism in the β_2-adrenergic receptor (*ADRB2*) promoter (Arg19>Cys) has been studied extensively. It is well established that *ADRB2* gene expression is controlled, in part, by a 19-amino acid peptide that regulates mRNA translation. This regulatory peptide is encoded by a short ORF (also called 5′-leader cistron [5′LC]) that is localized 102 bp upstream of the initiation translation codon (ATG). In transfected COS-7 cells with polymorphic constructs containing the 5′LC and complete *ADRB2* coding region, the expression of *ADRB2*, as measured with receptor density, was 72% higher in the 5′LC-Cys19 constructs than its counterpart Arg19, without marked differences in mRNA levels, indicating that the regulation of gene expression by uORF is principally at the translational level [20]. Furthermore, in human airway smooth muscle cells, when compared with the Arg19, the Cys19 has approximately twofold higher *ADRB2* expression [20] and significantly lower acute and long-term desensitization of isoproterenol-induced cyclic adenosine monophosphate (cAMP) production [21].

In addition, the extent to which mutations in the uORFs lead to human disorders is unclear. Recently, a uORF polymorphism (Lys9→Glu) in human dopamine D3 receptor gene (*DRD3*) was found to be associated with schizophrenia [22].

Promoter sequence methylation

Normally unmethylated cytosine phosphoguanine dinucleotides (called CpG islands) exist in the promoter region of some genes. Recent studies have shown that hypermethylation of the CpG sites (and Sp-1 binding domains in some cases) is involved in suppression of gene expression, principally at the transcriptional level [23]. Some evidence has indicated that aberrant promoter methylation is a common and early event in cancers, affecting a variety of genes with diverse function, and that inactivation of certain genes through CpG methylation may play an important role in the pathogenesis of some tumors, such as prostate cancer and breast cancer. For example, the gene π-class glutathione-*S*-transferase (*GSTP1*) promoter hypermethylation is seen with significantly greater frequency in patients with prostate cancer than in those with benign prostate hypertrophy [24].

Intronic mutations

The intron is defined as a segment of noncoding DNA present between two exons of a gene. In general, mature mRNA is formed when intronic segments are spliced out and assembled together with 5′-UTR, all exons, and 3′-UTR. Intronic variants can be categorized as two groups accordingly, splicing variants (within 8 bp of intron) and nonsplicing variants (deep within intron) [2].

Premature terminator codon & nonsense-mediated mRNA decay

mRNA often contains premature termination codons (PTC, or nonsense codon) as a consequence of mutations and RNA splicing errors. It is estimated that up to 30% of the mutations that cause human diseases contain PTCs that result in truncated C-terminal products and premature signaling for the termination of translation [25–27] through rapid decay of the mRNA containing nonsense mutations [28,29] or through exon-skipping during the period of pre-mRNA splicing [30]. For the drug disposition genes, the *CYP3A5*3* variant is a good example. The allele, a 6986A>G transition within intron 3 of the gene *CYP3A5*, creates a cryptic acceptor splice site in its pre-mRNA, resulting in the incorporation of 131 bp of intron 3 sequence (called pseudoexon 3B) in the matured mRNA and involving subsequently improperly spliced mRNA, a frame-shift, and finally producing PTC [31]. For the human liver microsomes genotyped with *CYP3A5*3*, there is an eightfold higher total RNA level in the group bearing *CYP3A5*1/*3* than *CYP3A5*3/*3* [32]. This suggests that the low-level expression of *CYP3A5*3* may partially be the result of the nonsense-mediated mRNA decay (NMD) pathway [31], a general mRNA surveillance mechanism. Consistent with an earlier prediction [31], mRNA of splicing variants containing pseudoexon 3B is more unstable than that of *CYP3A5*1* [33].

Nonsplicing variants, or mid-intronic or deep-intronic variants

Nonsplicing variants refer to mutations that are localized neither at the splice sites (that is, 'gt' in the donor site, and 'ag' in the acceptor site) nor at the exon–intron boundaries (also called junctions). In theory, these variants should not possess functional significance or clinical relevance, since they have been spliced out of mRNA or cDNA sequences. In fact, some nonsplicing variants have been associated with altered expression and function of a gene and variable drug response. An

often-cited example involves a common intronic variant of the cholesteryl ester transfer protein (*CETP*) gene and its relationship to the progression of coronary atherosclerosis [34], in which the polymorphism of interest was denoted as the presence or absence of a restriction site for enzyme *Taq*I in intron 1 of the *CETP* gene and this common variant appears to be able to predict whether men with coronary artery disease will benefit from treatment with pravastatin to delay the progression of disease. Another example relates to genetic prediction of the maximum doses of the anti-epileptic drugs, carbamazepine and phenytoin [35]. It is well established that voltage-sensitive sodium channels in neurons (SCNs) are the common target of such drugs. In this retrospective cohort study [35], an intronic polymorphism in the *SCN1A* gene (IVS5-91G>A) was associated with markedly reduced maximum dose requirements of both drugs, confirming a functional replication of the effects of the variant *SCN1A* gene. In contrast, large-scale population studies were available showing that intronic variants in the gene (which encodes corticotropin-releasing hormone receptor 1 [CRHR1]) are consistently associated with greater response to inhaled corticosteroids in each of three asthmatic populations [36]. Clearly, it is more likely that these nonsplicing variants may just represent imperfectly correlated genetic markers in strong linkage disequilibrium with actual causal variants of the tested genes or other functionally closely linked gene(s).

Variation in the 3′-untranslated regions

The 3′-UTR, located downstream of the coding sequences, is an important structural component of the full-length cDNA. It has been demonstrated that this region can also modulate gene expression [37]. A recent study has well characterized the importance of the gene 3′-UTR [38], in which introduction of the 3′-UTR region of the human *ADRB2* gene into the 3′-UTR of a heterologous receptor resulted in a 70% decrease in luciferase activity without marked alterations in luciferase mRNA levels, whereas deletion of its 3′-UTR sequence led to roughly twofold increase in receptor expression. Moreover, when transfected with full-length receptor cDNA, the receptor transcripts were distributed between polysomal and nonpolysomal fractions. After deletion of 3′-UTR sequence from the *ADRB2* cDNA, the distribution of receptor mRNA shifted clearly toward the polysomal fractions, suggesting increased translation. This example has definitely documented that the 3′-UTR sequences can suppress the translation of some (if not all) genes to certain extent, presumably by affecting their mRNA localization.

Within the 3′-UTR, the poly(A) tail is an important element influencing mRNA stability and its translational control [39,40]. Removal of the poly(A) tail from the 3′ end of capped mRNA decreases translation [41]. One of the mechanisms underlying this is that the poly(A) tail can increase the stability of an mRNA molecule by protecting the mRNA from digestion by 3′→5′ exonucleases [42]. A second common domain is a *cis*-acting adenine-uridine-rich element (ARE) in the 3′UTR, which is known to be associated with mRNA degradation, and even agonist-induced ADRB2 desensitization [43], suppressing gene expression at the translational level. However, few mutations have been identified in these domains. To date, few 3′-UTR-associated diseases have been characterized. Typical cases include:

- A polymorphism in the 3′-UTR of C-type natriuretic peptide gene that has been associated with hypertension [44]
- A G>A transition in the 3′-UTR region of the prothrombin gene, resulting in the genetic disorder of familial thrombophilia [45]

Summary

The transcription regulatory regions constitute a small fraction of roughly 95% of the human genome that does not encode proteins. However, such regions may determine the chronology, location and level of gene expression under control of splicing, transcription, and translation. Functional polymorphisms in such regions may lead to altered transcript biogenesis and metabolism, including processing, trafficking, stability, and translation control. Abnormal protein biogenesis may result from truncated products (nonsense mutations), splicing changes, frame-shift mutations, missense mutations, and insertion/deletion in exons, and abnormal protein trafficking.

Highlights

- The functionally significant variants in the noncoding regions of genes are frequently identified in the upstream open reading frames, the CpG islands, binding sites of transcription factors or nuclear receptors, splicing sites, the poly(A) tails and adenine-uridine-rich elements.
- In addition to exonic mutations, some noncoding variants also lead to altered gene expression and function.

Table 1. Major examples of the functionally significant noncoding variants.

Variants (gene)	Phenotype	Ref.
Haplotype group A (*VKORC1*)	Associated with lower warfarin dose requirements	[3]
Haplotype 4 (*ADRA2B*)	Reduced maximal response to dexmedetomidine	[5]
HapB (*SCN5A*)	Slowed cardiac conduction	[6]
VNTR (n = 3,4,6) (*ALOX5*)	Nonresponse to ALOX5 inhibitors	[15]
Variant Cys19 (*ADRB2*)	Reduced desensitization	[21]
CYP3A5 3*	mRNA instability	[33]
Allele B1 (*CETP*)	Greater progression of diffuse atherosclerosis	[34]
IVS5-91G>A (*SCN1A*)	Reduced maximum dose requirements of carbamazepine and phenytoin	[35]
GAT haplotype (*CRHR1*)	Enhanced pulmonary function response to inhaled corticosteroids	[36]

ADRA2B: Adrenergic receptor,α2B; ADRB2: Adrenergic receptor β2; ALOX5: Arachidonate 5-lipooxygenase; CETP: Cholesteryl ester transfer protein; CRHR1: Corticotropin releasing hormone receptor 1; CYP: Cytochrome P450; Hap: Haplotype; IVS: Intervening sequence; SCN5A: Sodium channel, voltage-gated, type V, α; VKORC: Vitamin K epoxide reductase complex; VNTR: Variable number of tandem repeat.

Despite the importance of these noncoding sequences in regulation of gene expression, the ability to identify, decipher and predict the functional significance of the regulatory elements and their variants is limited (Table 1). Due to the high probability of type I error (resulting in false-positive findings) in genotype–phenotype correlation studies, replication of relevant results will be required to confirm them. In addition, as a result of marked ethnic patterns of the distributions of some, if not all, variants, the genotyping data in one ethnic group cannot be extrapolated from those obtained in another. With the accumulation and integration of knowledge, studying noncoding polymorphisms and their functional significance will narrow the pieces of the puzzle of why disposition to disease and response to drug therapy vary between individuals when combined with knowledge of coding variants and their clinical relevance.

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