(5000 words maximum)

Title

Role of noncoding variants in cancer

Preface (100 words)

Tumor genomes contain numerous somatic sequence variants. These include single nucleotide mutations, small insertions and deletions and larger sequence rearrangements. A large majority of these variants occur in noncoding parts of the genome. Noncoding variants can effect gene expression to variable extents and may have major functional consequences causing tumor progression. Although most previous studies have focused on the identification of functional variants in protein-coding genes, many recent studies suggest that the repertoire of noncoding somatic variants contains driver events playing an important role in tumor growth. Furthermore, numerous noncoding germline variants are known to play a role in cancer susceptibility. In many instances, tumor growth relies on an intricate balance between inherited germline and acquired somatic variants. In this review, we discuss the current understanding of the role of noncoding somatic and germline variants in cancer.

Introduction

The first tumor whole-genome was sequenced in 2008 (REF). As a result of the decreasing costs, whole-genomes of thousands of tumors have since been sequenced. The numbers of cancer patients that have undergone whole-genome sequencing (WGS) is only going to increase as precision medicine approaches are increasingly being adopted in the clinic (REF). Most of the variants obtained from WGS of tumor genomes lie in noncoding regions (Figure 1). In this review we provide an overview of the current understanding of the role of noncoding sequence variants in cancer development and growth. We note that most previous studies of somatic cancer variants have focused on exomes. However, there is an increased realization of the importance of noncoding variants in cancer and an ongoing collaboration between TCGA (The Cancer Genome Atlas) and ICGC (International Cancer Genome Consortium), called Pan-Cancer Analysis of Whole Genomes (PCAWG), aims to identify noncoding mutations of functional consequence in ~2500 tumor and matched normal whole-genomes.

Genetic susceptibility for complex disorders has been probed previously by numerous genome-wide association studies (GWAS). These studies have revealed that most loci associated with complex traits lie in noncoding regions of the genome (REF). Many studies have also explored the link between inherited germline variants and cancer susceptibility. In agreement with other complex traits, these studies also revealed many noncoding loci associated with altered cancer risk (REF eg from Francesca). Thus, noncoding regions play an important role in cancer not only due to the somatic aberrations in tumor cells, but also the inherited germline variants they contain. In this review, we also discuss germline variants that have been associated with increased cancer susceptibility, specially the cases where there is an intricate relationship between germline polymorphisms and somatic variants.

Besides sequence alterations, other changes in the noncoding regions such as epigenetic and transcriptional variation can also influence cancer development. For example, many noncoding RNAs are known to be misregulated in various cancers (REF), H3K4me1 sites can be lost or gained in cancer cells relative to matched normal (REF), etc. However, in this review, we focus on effects of DNA sequence variants in noncoding regions and suggest reviews such as XX and XX for discussions of other cancer associated changes.

Before we go into the details of effects of sequence variants in noncoding regions, we first provide brief overviews of the various noncoding annotations and different kinds of sequence variants.

Noncoding annotations

The noncoding parts of the genome were once thought to be junk DNA but are now well known to contain many different types of regulatory elements that modulate expression of protein-coding genes. These elements are generally identified by sequence conservation or functional genomics approaches and often display cell- and tissue-type specificity (Figure 2). Several large-scale efforts such as ENCODE (Encyclopedia of DNA Elements) [1] and the NIH Roadmap Epigenomics Mapping Consortium[2] have been launched to create a comprehensive map of these regions. These efforts aim to provide genome-wide functional annotations across multiple cell- and tissue-types.

The various classes of noncoding annotations are identified using several functional genomics assays. For example, DNase I hypersensitivity for regions of open chromatin, ChIP-Seq for binding peaks of transcription factors (TFs) and histone marks, RNA-Seq for noncoding RNAs, etc. Evolutionary conservation of genomic sequence is also used to annotate noncoding regions[3, 4]. The dynamic annotation of these regions across various cellular states may be thought as turning gene regulation switches on and off using epigenetic marks. For example, as shown in the schematic in Figure 2, differential H3K27ac marks across various tissues indicate variable enhancer loci although the sequence at these loci where TFs bind stays the same. As a result, sequence variants in these loci are likely to exhibit tissue-specific effects on gene expression. This makes the functional interpretation of noncoding variants even more complex.

Linking the linear noncoding functional elements to their target protein-coding genes is of great importance and crucial to understand the effects of sequence variants in them. Multiple approaches are used to link cis-regulatory regions to their target genes. For example: different variations of chromosome conformation capture (3C) technology [5, 6], correlation of transcription factor (TF) binding and expression across multiple cell lines [7], etc. The resulting linkages can then be studied as a comprehensive regulatory network [8] (Figure 2).

We summarize the various sources of noncoding annotations with the web links for file downloads in Table 1.

Genomic sequence variants

DNA sequence variants range from single nucleotide variants (SNVs) to small insertions and deletions less than 50bp in length (indels) to larger structural variants (SVs). SVs comprise of deletions and duplications that lead to copy-number aberrations and inversions and translocations that are copy-number neutral. An average human genome contains roughly 3 million sequence variants relative to the reference human genome [9], while a tumor genome contains thousands of variants relative to the germline DNA (Figure 1)[10]. Unlike germline variants, somatic variants arise during mitotic cell divisions. Due to their different biological origins, somatic mutations tend to show distinct genomic patterns than germline variants. For example: (i) A higher fraction of somatic variants contain large genomic rearrangements. Recurrent fusion events between distant genes have been observed in many cancer types but are relatively rare in germline sequences (REF; confirm this is correct). Complex genomic rearrangements including chromoplexy[11] and chromothripsis[12] are known to occur in cancer cells. Chromosomal aneuploidy, where an entire chromosome may be lost or gained, is also often observed in cancer (REF). (ii) Somatic sequence variants may not be shared by all cells in the tumor tissue due to clonal evolution (REF). Such tumor heterogeneity makes interpretation of somatic variants more complex. (iii) Various phenomena, such as kataegis (localized hypermutation)[13] and other mutational signatures[10] are characteristic only of somatic variants.

Known cases of somatic variants playing a role in tumor development and growth

Somatic variants can effect gene expression in many different ways, e.g. point mutations in binding motifs of sequence-specific TFs may disrupt their binding, large deletions may delete entire TF binding sites/enhancer elements, etc (Figure 3). In this section, we discuss some known cases of somatic variants and their likely role in oncogenesis. We note that Vogelstein et al introduced the concept of Mut-driver and Epi-driver protein-coding genes, those that contain driver mutations and those that show aberrant expression providing selective growth advantage due to epigenetic changes, respectively[14]. Here we introduce an additional category, NcMut-driver genes, those that show aberrant expression providing selective growth advantage due to mutations in their noncoding regulatory regions. The examples discussed below correspond to such NcMut-driver genes. Different noncoding elements are effected by somatic changes --

a) Promoters: Recurrent mutations have been observed in the promoter of the *TERT* gene in many different cancer types[15-18]. These mutations create binding motifs for Ets/TCF TF leading to its binding and subsequent up-regulation of *TERT* (Figure XX). Tumors in tissues with relatively low rates of self-renewal (including melanomas, urothelial carcinomas and medulloblastomas) tend to exhibit higher frequencies of *TERT* promoter mutations[17]. The high occurrence of these mutations points to their role as drivers as opposed to passengers.
b) Enhancers: Enhancers constitute important cis-regulatory elements and play a major role in gene transcription. Super-enhancers are regions that recruit many TFs and drive expression of genes that define cell identity[19]. Recently, it was reported that somatic mutations create MYB binding motifs in T-cell acute lymphoblastic leukemia (T-ALL) which results in formation of a super-enhancer upstream of the *TAL1* oncogene resulting in its overexpression [20]. In another study, it was reported that somatic SVs juxtapose coding sequences of *GFI1* or *GFI2* proximal to active enhancers (called 'enhancer-hijacking') in

medulloblastoma [21] (Figure XX). In this case, although the SV effects the coding sequence, its functional impact occurs due to the activity of the enhancer region.

c) UTRs: Genomic lesions hitting UTRs are also known to be associated with cancer. The 5' UTR of *TMPRSS2* is frequently fused with Ets genes (*ERG* and *ETV1*) in prostate cancer [22]. This leads to *ERG* overexpression further disrupting androgen receptor (AR) signaling.

d) Other TF binding sites: Genomic rearrangements significantly associated with androgen receptor binding sites in a subset of prostate cancers [23, 24]. Basically this shows that AR binding drives the formation of structural rearrangements in some sub-types of cancer. [[To MG: I don't think this shows the functional role of noncoding sequence variants – infact noncoding sequence variants are the result of AR binding there. So I think we should exclude but need to discuss with Mark R.]]

e) Noncoding RNAs (ncRNAs) and their binding sites: Mis-regulation of ncRNAs is a cancer signature, and at least in some cases it could be due to the presence of somatic variants in them. For example, *MALAT1*, which is frequently up-regulated in cancer, was found to be significantly mutated in bladder cancer [25] and copy-number amplification of long ncRNA, lncUSMycN, is thought to contribute to neuroblastoma progression [26, 27]. In another scenario, pseudogene deletion can effect competition for miRNA binding with the parent gene, which in turn could effect expression of the parent gene. This is observed in certain cancers where *PTENP1* pseudogene is deleted, thereby leading to down-regulation of the parent *PTEN* tumor-suppressor gene [28] (Figure XX). Mutations in miRNA binding sites can also effect their binding, e.g. mutations in miR-31 binding site can lead to overexpression of AR in prostate cancer [29].

Germline variants in noncoding regions that alter cancer susceptibility or patient survival

Cancer is known to have a familial component and several loci associated with increased cancer risk have been identified by GWAS. Many of these loci lie in noncoding regions. Several cases of regulatory noncoding risk loci indicate that cancer results from a complex interplay of inherited germline and acquired somatic mutations. Unlike somatic variants, germline variants occur in all tissues of the body. However, their functional effect might not be manifested in all tissues, e.g. if they occur in regions of closed chromatin or if they disrupt a binding site of a TF that is not expressed in the tissue, etc. We discuss a few examples of noncoding germline variants related to cancer susceptibility here.

a) Promoters: Besides their somatic recurrence, germline mutations in *TERT* promoter are associated with familial melanoma[16]. Similar to the effect of somatic mutations, these mutations create binding motifs for Ets/TCF TFs. The functional effects of these mutations are more likely to be exhibited in the tissues where these TFs are expressed. Elevated expression of the TCF *ELK1* gene is observed in female specific tissues, such as ovary and placenta. Horn et al. reasoned that besides melanoma, this may be related to the increased ovarian cancer risk in women who are carriers of the mutation[16].

It was reported in another study that a common SNP (rs2853669) at another location in the *TERT* promoter modifies the effects of somatic *TERT* promoter mutations in bladder cancer on patient survival [30]. If the patients with somatic lesions in the *TERT* promoter carried this SNP,

they showed better survival. From a mechanistic viewpoint, the common SNP might weaken the effect of somatic mutations since it disrupts a pre-existing Ets2 binding site.

The multiple germline and somatic variants in the *TERT* promoter demonstrate their complex relationship with cancer susceptibility, oncogenesis and patient survival.

b) Enhancers: Multiple SNPs in a gene desert on chromosome 8q24 upstream of *MYC* are related with increased risk for many cancer types (breast, prostate, ovarian, colon and bladder cancers and chronic lymphocytic leukemia) [31]. Several observations, such as histone methylation and acetylation marks and 3C assays, suggest that these 8q24 SNPs occur in regions that act as enhancers for *MYC* in a tissue-specific manner. In another example, a prostate cancer risk associated SNP occurs in a cell-type specific enhancer and leads to increased *HOXB13* binding. This in turn upregulates *RFX6* and is linked to increased prostate cancer susceptibility [32].

(d) Noncoding RNAs (ncRNAs): While most cancer associated polymorphisms are related to increased risk, some of them can also be beneficial and reduce susceptibility. A SNP in miR-27a impairs the processing of pre-mir-27a to its mature version. The reduced miR-27a level results in increased expression of its target *HOXA10*, which reduces susceptibility to gastric cancer [33].

(e) Intronic splice site mutations: A rare mutation in the intron of *BRCA2* causes aberrant splicing and is related with Fanconi anemia (a rare recessive disorder involving high cancer risk) [34].

We note that the examples above do not include an exhaustive list of all known cases of noncoding germline variants associated with altered cancer risk, but are meant to illustrate the diverse ways in which many regulatory polymorphisms exhibit their functional effects. Various other methods of identifying variants with potential functional consequences, such as expression quantitative trait loci (eQTL) and allele-specific expression analyses, have been used to interpret GWAS cancer loci [35-37]. Such studies reveal germline determinants of gene expression in tumors and help establish a link between noncoding risk loci and their target coding genes.

The known examples of cancer risk loci illustrate that the effects of noncoding mutations and the interplay between germline and somatic variants can be complex. Furthermore, for hormone-regulated cancers (such as breast and prostate), the effects of altered hormonal generation during an individual's lifetime might be different depending on the germline genotypes of other genes and regulatory elements in the hormone-regulated pathway (Supplementary Figure XX) [To confirm with Francesca]. All these cases show that the effects of somatic mutations on tumorigenesis depend on the existing germline variants and their binary classification into drivers and passengers does not capture this complexity.

Different types of cancer

Somatic mutation frequency varies considerably across different cancer types [10, 38]. In general, slow growing tumors, such as carcinoid tumors and prostate cancer, harbor fewer mutations as compared to rapidly growing melanomas, bladder cancer and lung cancer. However, growth rate is not the only determinant and some rapidly growing tumors, such as acute myeloid leukemia (AML), Ewing sarcoma and neuroblastoma, are on the lower spectrum of somatic mutations. Many slow growing tumors have canonical oncogenic drivers that might obviate the need for a cancer to acquire mutations as a mechanism for selective advantage. Specifically, some of the tumors listed with the lowest mutations rate harbor defining genomic alterations and gene fusions: rhabdoid tumors harbor SMARCB1 deletions, Ewing sarcoma harbor a recurrent ETS gene fusion (EWS-FLI1), thyroid cancers harbor common RET mutations and fusions RET/PTC1, neuroblastomas harbor amplification of NMYC, and prostate cancers harbor common ETS gene fusions (most commonly TMPRSS2-ERG). We expect most mutations in tumors with high total numbers of mutations to be passenger events with no functional consequence. We also expect that a higher fraction of noncoding mutations would be passengers with little or no functional consequence as compared to coding mutations. In agreement with this hypothesis, we observe that the fraction of noncoding mutations is positively correlated with the total numbers of mutations across 11 (or 12) cancer types (Figure XX; Spearman correlation between total number of mutations and noncoding fraction=0.32, p val=2.20e-15). [To MG: I think we should include in Figure caption that this result is when we exclude pilocytic astrocytoma which shows a lot of variability in number of mutations and has been hypothesized to be a single pathway disease]

OK

[[To MG: BELOW TEXT IS STILL IN BULLET POINTS]]

Computational methods to identify noncoding somatic variants with functional consequences

(a) Discussion of currently available computational methods to predict noncoding driver mutations from whole-genome sequencing data, for example, FunSeq [4], CADD [39] and GWAVA [40]. We will also list these in Table 3 with associated website links.

Experimental approaches to understand the functional effects of noncoding mutations

Finally, we will discuss experimental ways to test which noncoding mutations have functional effects (e.g. genome editing using CRISPR, luciferase reporter assays, high-throughput assays, etc). We will also discuss the scale and approximate cost of all the techniques and summarize them in Figure 4.

Conclusions/perspective

Recent studies have shown that small changes in gene expression caused by noncoding mutations can have large phenotypic impact (e.g. a SNP in enhancer causing 20% change in *KITLG* expression is responsible for blond hair color[41]). We postulate that the combined effect of

small changes in expression due to noncoding mutations in cancer might be huge. Under this notion, genomic variants contribute to oncogenesis with varying probabilities, as opposed to the binary classification of mutations into drivers and passengers. While some somatic variants may have a direct role (such as *TERT* promoter mutations found in many different cancer types[17]), others may indirectly modulate important cancer pathways (such as genomic rearrangements perturbing androgen receptor binding sites in a subset of prostate cancers[23, 24]).

(a) Cancer arises because of accumulation of multiple driver mutations[14] -- some of these drivers could be noncoding. There is a bias in the literature for driver noncoding mutations because people haven't explored these regions to the same extent as coding genes, for example, the majority of TCGA studies have focused on exomes.

(b) There is a debate in the community about whether we should analyze whole-genomes vs exomes. Studies of somatic noncoding mutations are currently mostly for research purposes, as opposed to regular clinical use. This is primarily because current therapeutic approaches attempt to target proteins. It is possible that alternate methodologies, such as genome editing using CRISPR, may be used in future (e.g. CRISPR/Cas9 mediated editing has been used for HIV in cell lines [42] and muscular dystrophy in mice [43]). However, noncoding germline variants associated with increased cancer susceptibility should be important for risk assessment and potentially for preventive approaches.

(c) In relation to (b), it is very important to know the links between cis-regulatory regions and their target genes. Although many approaches exist (as discussed under 'Main sections'), this remains a very active and important area of research, especially the development of high-throughput choromosomal capture technologies.

(d) Even when the links between regulatory regions and target genes are known, it is important to study effects of mutations in all elements controlling gene expression – thus network approaches will be important to understand the role of noncoding mutations in cancer. We might also be able to identify new pathways or novel participants in known pathways that are important in cancer.

Glossary

Possible Glossary terms

Germline variants
Somatic variants
Cis-regulatory regions

Proposed display items

Figure 1: Numbers of total and noncoding vs coding mutations for different cancer types (Yao)
Figure 2: Noncoding annotations (Ekta)
Figure 3: Effect of sequence variants in noncoding regions in oncogenesis (Ekta)

Figure 4: Experimental approaches used to understand the functional effects of noncoding variants (Dimple)

Table 1: Noncoding annotations (include FANTOM)
Table 2: Computational methods to prioritize noncoding mutations with functional effects

Supplementary Figures
Figure from Francesca

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