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There is a world beyond protein mutations: The role of non-coding RNAs in melanomagenesis

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Abstract

Until recently, the general perception has been that mutations in protein coding genes are responsible for tumorigenesis. With the discovery of ^{V600E}BRAF in about 50% of cutaneous melanomas there was an increased effort to find additional mutations. However, mutations characterized in melanoma to date cannot account for the development of all melanomas. With the discovery of microRNAs as important players in melanomagenesis protein mutations are no longer considered the sole drivers of tumors. Recent research findings have expanded the view for tumor initiation and progression to additional non-coding RNAs. The data suggest that tumorigenesis is likely an interplay between mutated proteins and deregulation of non-coding RNAs in the cell with an additional role of the tumor environment. With the exception of microRNAs, our knowledge of the role of non-coding RNAs in melanoma is in its infancy. Using few examples we will summarize some of the roles of non-coding RNAs in tumorigenesis. Thus, there is a whole world beyond protein coding sequences and microRNAs, which can cause melanoma.

Keywords

melanoma; non-coding RNA; microRNA; epigenetic modifications; alternative splicing

Introduction

Melanoma is one of the few cancers with increasing incidence and deaths over the last decades (*Cancer Facts & Figures 2012*, Atlanta: American Cancer Society 2012, (1)). There is a massive effort to determine the drivers of cancers and their genetic signatures to understand the biology of tumorigenesis and to identify new therapeutic targets. Although there is no doubt that the tumor microenvironment has a significant influence in tumorigenesis (2–4), we will focus on the role of genetic changes and RNA expression in tumor cells.

Mutations do not explain all tumors

About 50% of cutaneous melanomas harbor a ^{V600E}BRAF mutation (5, 6) followed by 15% that have a mutation in *NRAS*. In familial melanoma, which accounts for about 10% of melanomas, 40% of the patients have a mutation in *CDKN2* (7). Additional mutations, like in *C-Kit*, are found in melanomas in less numerous cases (8). Although the ^{V600E}BRAF

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mutation is the most common mutation in cutaneous melanomas, it is rarely seen in uveal melanomas, those of internal organs (9) or other types of skin cancers. Instead, metastasizing uveal melanomas have a high frequency of *BAP1, GNAQ* and *GNA11* mutations (10, 11).

Despite the high frequency of the above described mutations, they are not sufficient by themselves to induce cancer. For example nevi carry the ^{V600E}BRAF mutation without any signs of malignant transformation (12). The same is true for *NRAS*. A mutation in this gene alone is not sufficient to induce tumors (13). These findings confirm the notion that a second mutation/alteration or even a third is necessary for the transformation of cells, which reflects Knudson's original two hit theory (14, 15). Using the newest development in 2nd generation sequencing, several additionally mutated genes have been determined in melanomas (reviewed in (8)). The approaches ranged from sequencing just the tyrosine kinome (16), whole exome sequencing (17), whole genome sequencing (18, 19) to transcriptome sequencing (20). Even with these additionally determined mutations we are not able to explain all tumor cases in melanoma. Obviously we are still missing important players in the induction of cellular transformation. One limitation of all the above mentioned studies has been their focus on mutations in protein coding regions of the genome (8). Other mechanisms have been neglected. For example, although the ^{V600E}BRAF mutation is common in thyroid cancer, BRAF can also be activated by alternative splicing (21) without the V600E mutation. This alteration would have been most likely missed by solely testing for mutations in the protein coding region. A similar instance can be seen in the resistance to Vemurafenib, a ^{V600E}BRAF specific inhibitor (22). Resistance to this drug develops in most treated melanoma patients over time and one of the described resistance mechanism is alternative splicing of ^{V600E}BRAF (23). Again alternative splicing can have a similar effect as the introduction of a mutation. Based on these results and others, one has to reconsider if tumorigenesis relies solely on mutations in proteins. This raises the question, if additional genomic and expression modifications occur, which could explain aberrant gene expression in tumor cells?

A comprehensive analysis of a malignant melanoma showed that actually the minority of somatic mutations (~1.5% substitution mutations including the UTR region) was found in regions corresponding to mature mRNAs. The majority (~98.5%) of mutations were in other regions of the genome (18). We also know that only a small part of the genome codes for proteins (~2%) and that the majority of RNA is transcribed from non-coding regions (24, 25). The newest data release of ENCODE confirmed that the majority of the genome is transcribed (26). Therefore, our focus on the role of coding genes in tumorigenesis disproportionally favors mRNAs and neglects the influence of non-coding RNAs (ncRNAs). The effects of ncRNAs range from influencing RNA stability, selection of splice variants to general transcription regulation, which either acts *in cis* by the complementary antisense RNA or *in trans* like microRNAs (miRNAs). The latter effect includes also long non-coding (lnc) RNAs which can modify the activity of promoters by epigenetic changes (24). An increasing amount of data describe the important role that ncRNAs play in tumors (27). Figure 1 shows different interactions where ncRNAs could influence the expression of mRNAs. Any dysregulation of mRNA by either changing its expression or splice variation could act like an oncogenic event pointing to the significance of ncRNAs and their role in tumorigenesis.

Non-coding RNAs are important in tumorigenesis

microRNAs contribute to melanoma development

The best studied group of non-coding RNAs are miRNAs, which in most cases decrease targeted mRNA levels (28). For a general review of miRNAs in cancer see reference (29).

miRNAs can act as tumor suppressors as well as oncogenes (30) and there is no doubt about their role in tumorigenesis (31). In tumors the biogenesis of miRNAs is disturbed (32, 33), which will alter the expression levels of miRNAs and ultimately the expression of genes regulated by miRNAs (32). In melanoma, the miRNAome (34) has been determined and the role of miRNAs in melanomas has been reviewed (35–37). miRNAs are involved in all steps of tumorigenesis from initiation (38) to metastasis (39, 40). Melanoma subtypes differ in their miRNA signatures (41), which can serve as a prognostic biomarker (42). Additionally, miRNAs not only regulate mRNAs but also other ncRNAs (43, 44) and they themselves are epigenetically regulated (45). This places ncRNAs in a wider, multilayer regulatory network of transcriptional and translational control (Fig. 2).

Do lncRNAs have a role in melanomagenesis?

Besides miRNAs, there are also lncRNAs (46). As implied by their name, they are larger than miRNAs with a minimum length of 200bp and up to several kilobases. One of the best studied members is *XIST*, which is involved in the inactivation of the X chromosome (47). lncRNAs are divided in three major groups: long intergenic non-coding RNAs (lincRNA), which are located away from protein coding regions, antisense RNAs (asRNA), which are transcribed in reverse orientation and overlap with a known gene, and intronic lncRNAs. The groups have partly overlapping activities. For example, members of each group regulate epigenetic modifications, however only the transcription of asRNAs directly interferes with transcription of its corresponding sense counterpart by polymerase stalling. The different functions attributed to lncRNAs (reviewed in (48, 49)) include control of pluripotency and differentiation in stem cells (50), setting of epigenetic marks (51), and functioning as enhancer RNAs (52). One subgroup of intron-derived lncRNAs, the sno-lncRNAs, is associated with changes in splicing (53). Although lncRNAs are expressed in normal cells with tissue-specific expression patterns, aberrant expression occurs in tumors (54) and they play roles in cancer progression (55). Based on the expanding knowledge of their regulatory influence lncRNAs may represent “a new frontier of translational research” in cancer (56). There is very limited information on the role of specific lncRNAs in melanoma. In 2011, Khaitan *et al.*(57) showed that the lncRNA SPRY4-IT1 modulates cell growth and differentiation and its knock-down increased apoptosis. Flockhart *et al.*(58) described the effect of ^{V600E}BRAF on the expression of about 100 lncRNAs.

The number of known lncRNAs is still increasing. The subclass lincRNAs alone contains over 8,000 members (59) with different activities. One of the described functions of lnc RNAs is setting of epigenetic marks. For example, the lncRNA *HOTAIR* is involved in the setting of epigenetic marks by the polycomb repressive complex 2 and its expression levels are increased in breast tumors. A high expression level implies a poor prognosis for metastasis and survival in breast tumor patients (60). It is well established that epigenetic changes occur in tumors (61, 62), and that these changes play a role in melanomas as well (63). Interestingly, conditions which are indirectly linked to the onset of tumors, such as stress (64, 65) and age (66), induce epigenetic changes. Issa and Garber suggested the presence of an epigenetic predisposition to cancer (67). Therefore, epigenetic changes induced by dysregulation of ncRNAs may act like an oncogenic event. One has to emphasize that different non-coding RNAs, lincRNAs, asRNAs (68) as well as miRNAs can induce epigenetic changes, but at the same time miRNAs are regulated by epigenetic changes (45).

The next group of lncRNAs are asRNAs, which not only contain non-coding RNAs but also coding RNAs although to a lesser extent (e.g. *FGF-2* and its asRNA *FGF-AS* (69)). AsRNAs are relatively common (70, 71). They regulate the expression of their corresponding sense genes by different mechanisms (72), like influencing sense RNA stability, epigenetic changes and alternative splicing (73, 74). The idea that splicing is a

factor in tumorigenesis is not new (75, 76), but the study and detection of splice variants was previously hampered by technical limitations. With increasing numbers of high-throughput RNA sequencing studies completed (reviewed in (77)), we will get a more complete list of splice variations (78) between normal and diseased tissues and thus a better understanding of their role in cancer, including melanoma. In mice, melanocytes and melanomas differ in splice variants (79). Even more astonishing is the observation that breast cancer cells grown in 2D or 3D culture differ in their splice variants (80), which suggests that alternative splicing can occur in response to subtle changes in the microenvironment. In melanoma, different splice isoforms of *MITF,* the master regulator of melanocyte development, have been described (81) as well as aberrant splicing of the tumor suppressor *Bin1* (82). Splicing even plays a role in resistance to cancer treatments such as Vemurafenib (23). Based on these findings, targeting specific splice variants by antisense oligonucleotides has been suggested for cancer therapy (83) and splice variants have been proposed as cancer biomarkers (84).

Alternative splicing is not an isolated incident. Splice variants of DNA methyltransferases (85) differ in their activity, which may result in epigenetic changes. At the same time epigenetic changes regulate alternative splicing (86). Additionally, changes in the 3'UTR of mRNAs due to alternative splicing can alter the recognition by miRNAs (87). Thus, alternative splicing is part of a larger interacting network and is influenced by ncRNAs (88).

Minor modulation of mRNA levels can have a significant effect

In any discussion about the role of ncRNAs, one has to be aware that already small expression level changes can have an effect. One of the best examples is the tumor suppressor PTEN (89). Pandolfi's group (90, 91) has demonstrated that small changes in the expression levels of *PTEN* are sufficient to achieve an effect in tumor progression, thus, complete deletion is not necessary. It is known that asRNA levels influence sense RNA levels. Epigenetic changes are another way to vary RNA expression. The discovery of ceRNAs (competing endogenous mRNAs), which influence mRNA levels by competing for miRNAs, adds one more mechanism to modulate the expression level of a certain gene (92–94).

Are non-coding RNAs the new frontier in melanoma research?

Biological and bioinformatics analyses of non-coding regions are still in their infancy, with the exception of the well-established miRNA field. The cancer genome atlas is just the beginning and much research remains to be done. We will gather much information on exon sequences, genomic deletions and amplifications, but like first-graders we still lack the knowledge to fully comprehend the information contained in the whole genomic sequence. We still do not know how to interpret the sequences and possible mutations of non-coding RNAs. In contrast to protein coding sequences, no reference sequences exist for those regions to define wild type versus mutated versions. Because the functions of most ncRNAs are unknown, no assays are available to determine, which sequence changes influence their function.

Analyzing each single compartment (protein, mRNA, ncRNA) and their interactions is already a major challenge. However, the analyses of the key players are further hindered by the fact that splice variants, epigenetic changes and ncRNAs interact and influence each other. For example, histone modifications regulate alternative splicing (86) and conversely, splice variants of DNA methyltransferases result in epigenetic changes (85). All these data stress the fact that control of mRNA expression (transcription factors, translation, etc.) is overlaid and controlled by an interacting network of ncRNAs (Fig. 2). Any disturbance at any level, including ncRNA interactions, will eventually have consequences at the protein

level. This will either be reflected in the quantity of protein or in introduced modifications (e.g. alternative splicing).

Summary and perspectives

The discovery of ^{V600E}BRAF was a blessing and a curse for melanoma research. It gave the melanoma community a new target with impressive therapeutic results for patients, but at the same time resulted in neglect to study other areas, such as ncRNAs. We are aware that splice variants and asRNAs were *in vogue* some time ago and we are not the first to suggest studying them more carefully. It is time to return to old ideas using new tools (e.g. RNAseq). The overall fixation on mutations in coding genes as a cause of transformation limits us. It is time to combine both worlds, the coding as well as the non-coding RNAs, in our studies to understand the biology of cancer and to determine new targets in our battle with cancer. NcRNAs will be valid targets for tumor treatment and with the development of new RNA therapeutics we may be able to target ncRNAs more efficiently than with conventional antisense oligonucleotides (95). Additionally, ncRNAs may not be only direct targets. The results of Ingolia *et al.*(96) suggest that translation may occur from ncRNAs, which could provide new epitopes to develop tumor specific immunotherapies. In our own research, we have identified tumor specific T cell antigens encoded by asRNAs. Table 1 shows some examples of conceivable applications based on results gained by studying ncRNAs.

We are well aware that this viewpoint omits important aspects of tumor drivers such as the influence of the tumor microenvironment (2–4), and other modifications such as alternative initiation of translation on non-AUG codons (97) or RNA editing (98). Considering all the possible modifications which can occur in tumor cells, one may feel overwhelmed whether we will ever come close to full understanding and conquering cancer. On the other hand, with all these multilayer regulatory networks we may find new “weak spots” of tumors for intervention. We provided only a glimpse into the world of non-coding RNAs and their possible roles in tumorigenesis. We hope to inspire readers to expand their view beyond mutated proteins and to include non-coding RNAs in their studies. We apologize to all colleagues whose work we could not cite due to space restrictions.

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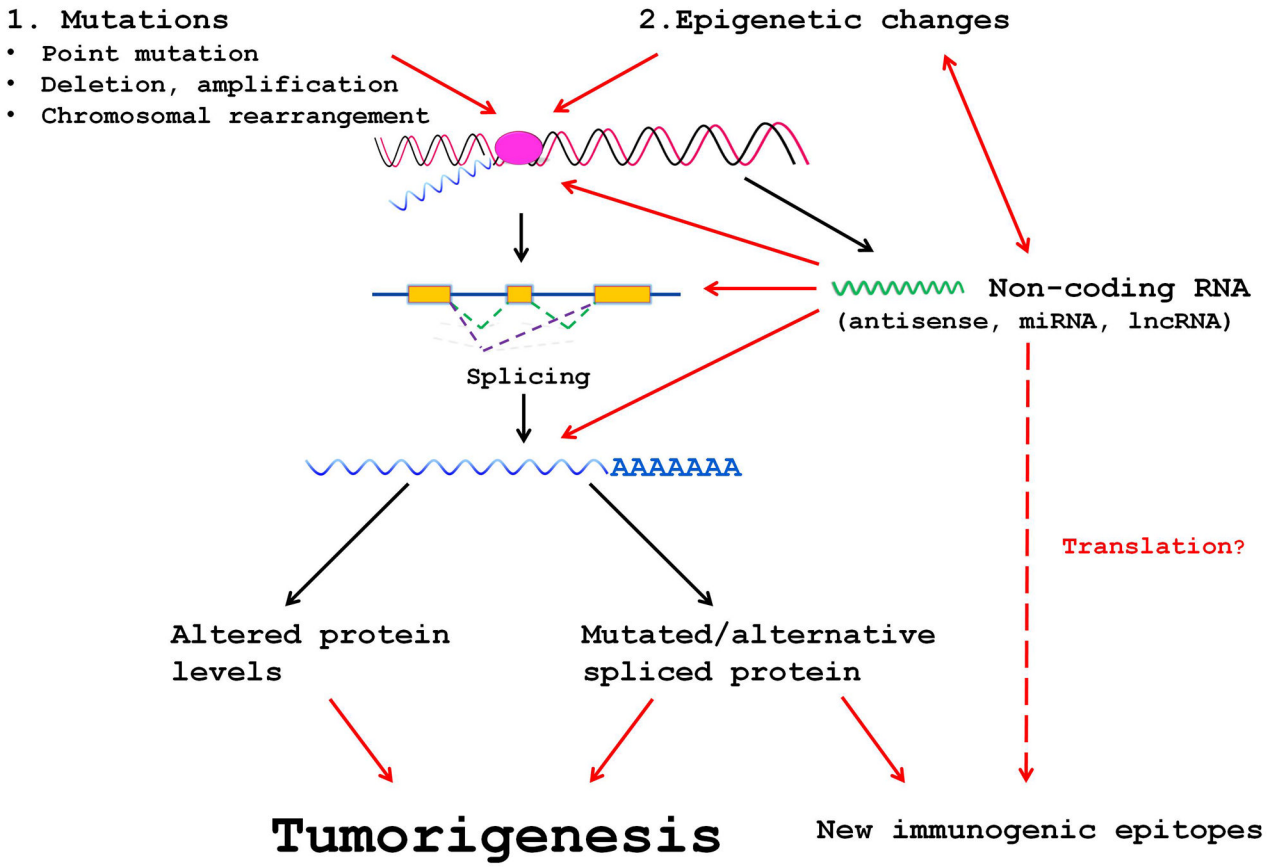

Fig. 1.

Possible interactions of non-coding RNAs with protein expression during tumor induction. Red arrows point to interferences with protein expression by the disturbed expression of non-coding RNAs.

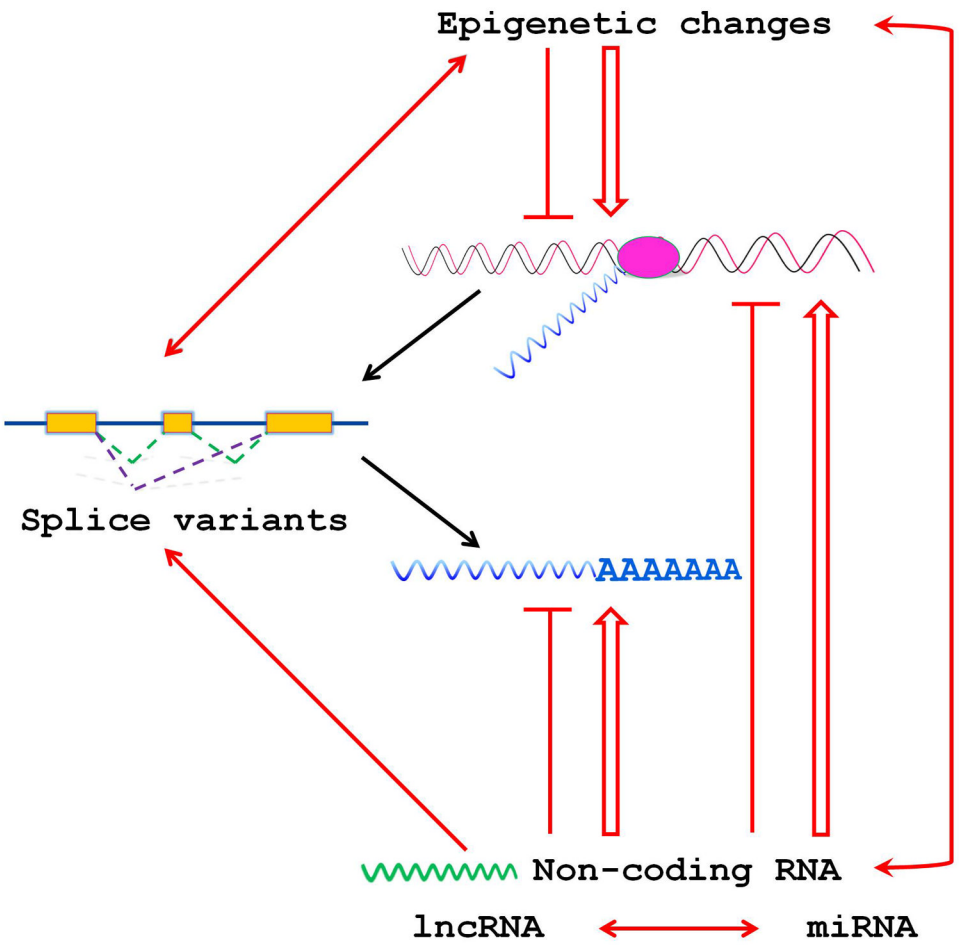

Fig. 2.
Simplified map of interactions in the non-coding RNA network itself and with mRNA expression. The symbols mean ⊥ inhibition, ⇑ stimulation, ⟷ mutual interaction.

Table 1

Conceivable applications based on non-coding RNAs.

- Diagnosis
  - Presence of specific non-coding RNAs as biomarker for tumor (e.g. metastasis)
  - Classification of tumor subgroups (e.g. metastasis risk)
  - Treatment selection
- Therapy
  - Drugs and RNA therapeutics targeting specifically splice variants
  - Targeting non-coding RNA
  - Targeting epigenetic machinery
  - Influencing RNA/protein levels
  - Immunotherapies based on epitopes derived from non-coding RNAs