Long non-coding RNAs drive metastatic progression in melanoma (Review)

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Abstract. Metastatic melanoma is the leading cause of skin-cancer related deaths and while in recent years some progress has been made with targeted therapies, there remains an urgent unmet need for novel therapeutic treatments and reliable diagnostic, prognostic and predictive biomarkers. The emergence of next generation sequencing (NGS) has seen a growing appreciation for the role played by non-coding genomic transcripts in regulating gene expression and by extension impacting on disease progression. The long non-coding RNAs (lncRNAs) represent the most enigmatic of these new regulatory molecules. Our understanding of how lncRNAs regulate biological functions and their importance to disease aetiology, while still limited, is rapidly improving, in particular with regards to their role in cancer. Herein we review the identification of several lncRNAs shown to impact on melanoma disease progression and discuss how these molecules are operating at the molecular level.

Contents

1. Introduction

Malignant melanoma (MM) has one of the fastest-rising incidences of all cancers (1). This, coupled with the poor prognosis for patients in stages III and IV of the disease, is causing a growing healthcare burden on society. At the molecular level, rather than being a single disease, melanoma comprises a heterogeneous group of disorders that harbour distinct aberrations in diverse cellular processes such as cell cycle regulation, cell signalling, cell adhesion, cell differentiation and apoptosis (2). Such heterogeneity suggests that multiple mechanisms are involved in disease aetiology and this is reflected in the contribution of both different mutations and differential gene and protein expression associated with MM development and progression (3,4).

Our understanding of genomics is currently undergoing a paradigm shift. The past decade has seen staggering advances in next generation sequencing (NGS), precipitating an explosion of genome-wide transcriptome studies, culminating in 2012 with the completion of the ENCODE project (5). Large scale cDNA sequencing and interrogation of whole chromosome tiling arrays across a variety of cell types reveal that >90% of genomic nucleotides are transcribed (6). Perhaps the most surprising discovery to emerge from these studies is that only a tiny percentage of the RNA transcripts synthesised are fated to code for protein, with the vast majority being comprised of non-coding RNA. This realisation eschews the central dogma of molecular biology (7) and has ushered in a new era of non-coding RNA research that is focused on unpicking the functional significance of these prevalent transcripts (8,9).

Non-coding RNAs can be broadly defined according to their size as either short (<200 nt) or long (>200 nt). Short non-coding RNAs are well-characterised, particularly those involved in the production and operation of gene expression machinery such as ribosomal RNA, transfer RNA, small-nuclear RNA and small-nucleolar RNA. More recently, short non-coding RNAs that function to regulate, rather than operate gene expression have been intensely investigated and can be further divided into three main subcategories: PIWI-associated RNAs, which regulate transposable elements, small interfering RNAs and microRNAs (miRNAs), which mediate post-transcriptional silencing of mRNA and the recently described transcription initiation RNAs, which appear to instigate gene expression at the promoter level. Of these, miRNAs are the best understood and function to regulate both fundamental processes in biology, such as proliferation, apoptosis and differentiation, while also contributing significantly to disease aetiology, most notably cancer (10), but also cardiovascular disease (11), stroke (12) and several neurological disorders (13,14). The functional role played by miRNAs in the establishment and metastatic progression of melanoma was recently reviewed (15) and

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compiling data from several groups has enabled the coupling of differential miRNA expression in MM to specific target protein modulation and critically downstream functional consequence for disease (16-24). Such studies represent an emerging area of research, indeed, the prevalence of miRNAs in the human transcriptome continues to expand as deep-sequencing technologies improve, with current estimates identifying ~9,000 small non-coding RNAs, ~1,100 of which are potentially functional miRNA transcripts (5). However, this number is dwarfed by the pervasiveness of the long non-coding RNA (lncRNA) transcripts, with GENCODE identifying a staggering 22,444 non-coding transcripts >200 nt in length and more recent studies estimating this number to be nearer 32,000 (25).

In contrast to miRNA, our understanding of lncRNA function is in its infancy, however, huge efforts are being made to fill this knowledge gap, particularly in relation to their role in disease. The importance of such study is underlined by what we do understand about a handful of lncRNAs involved in fundamental aspects of biology. Most notable of these are the lncRNAs *XIST*, which inactivates gene expression from the X-chromosome during dosage equalisation and *HOTAIR*, a lncRNA that acts *in trans* via the Polycomb repressor complex to regulate genes at a distance, intimating that lncRNA are capable of more complex modes of gene regulation (26,27). In parallel to their role in the regulation of cellular processes and akin to their smaller cousins, the miRNAs, it is the involvement of lncRNAs in the development and progression of cancer that drives much of the current research on these transcripts. This review will focus on recent advances made in the discovery of lncRNAs with a functional role in the progression of melanoma metastasis.

2. Long non-coding RNAs drive metastatic progression in melanoma

The prognosis for patients with early stage, localised melanoma is favourable, with 10-year survival rates approaching 90%, depending on Breslow thickness, ulceration and mitotic index (28). This prognosis deteriorates rapidly with the onset of regional metastasis (10-year survival of around 50%) and for patients with stage IV melanoma and distant metastasis, 5-year survival rates are <10% (29). Clearly, metastatic progression in melanoma severely impacts on patient health and there is an urgent need to expand our understanding of the mechanisms underpinning this process. Numerous studies investigating the functional role played by lncRNA in cancer suggest that they are functioning to regulate events intimately associated with the metastatic transition, such as cell migration and tissue invasion (30,31). Here we will review our current understanding of lncRNAs known to impact on melanoma and consider what we understand about the functional mechanisms involved.

HOTAIR. HOTAIR is a member of an exclusive, but growing, family of lncRNAs that have comparatively well-defined cellular functions, in this case the epigenetic regulation of gene expression. HOTAIR is transcribed from the HOXC cluster and interacts with the Polycomb repressive complex 2 (PRC2), which it then trafficks to the HOXD cluster, facilitating transcriptional silencing of this 40 kb region (27). In addition to regulating the expression of this specific genomic region, HOTAIR also appears to regulate the gene expression at hundreds of other genomic locations via an interaction with the LSD1/CoREST/REST complex, which enables recruitment of PRC2 and LSD1 to chromatin for coupled histone H3K27 methylation and K4 demethylation (32). With such a pivotal role in epigenetic modification and transcriptional activity, it is not surprising that HOTAIR expression is deregulated in numerous cancers, including breast, lung, colorectal, pancreatic, ovarian hepatocellular carcinoma and gastrointestinal stromal tumours (GIST) (33-42), indeed HOTAIR has been shown to reprogram the cancer epigenome in breast cancer towards a metastatic state, cementing its position as a molecule of huge importance in cancer biology (43).

In order to determine if HOTAIR and other lncRNAs known to be deregulated across a range of cancers are upregulated in metastatic melanoma, Tang *et al* carried out quantitative PCR (qPCR)-based expression profiling on matched primary melanoma versus lymph node tumour samples from three patients (44). These analyses revealed two things: i) HOTAIR expression is dramatically upregulated by ~100-fold in metastatic versus primary melanoma; and ii) several other lncRNAs (HULC, MALAT-1, MEG3, NEAT1 and UCA1), which are associated with metastatic progression across a range of cancers were not differently expressed in metastatic melanoma, suggesting that lncRNAs contribute to metastasis in a cancer-specific manner (44). Moreover, it appears that elevated HOTAIR expression is a determining factor for the metastatic state, at least *in vitro*, with siRNA-mediated depletion of HOTAIR in the metastatic melanoma cell line, A375, resulting in significant decreases in both cell motility and invasion. These properties underpin metastasis, which involves the destruction of the basement membrane and migration of the tumour cells into the connective tissues before spreading to the lymph nodes and distal sites. The basement membrane is largely comprised of type IV collagen, which is degraded by matrix metalloproteinases (MMPs), specifically MMP-2 and MMP-9 (45). Intriguingly, *in situ* zymography assays carried out by Tang *et al* (44) show that depletion of HOTAIR in A375 cells resulted in reduced activity of MMP-2 and MMP-9, suggesting a possible mechanism for *HOTAIR*-mediated metastasis (Fig. 1A), a theory supported by recent data indicating that MMP gene expression is heavily regulated at the epigenetic level (46). Considering *HOTAIR's* association with epigenetic reprogramming and given the potential use of epigenetic drugs that target MMPs to treat melanoma (47), there is an urgent need to develop a keener understanding of such mechanisms.

SPRY4-IT1. *HOTAIR* appears destined to be involved in the aetiology of the majority of cancers, melanoma included. However, it was not the first lncRNA to be associated with melanoma, this accolade goes to the *SPRY4-IT1* transcript, a lncRNA that is derived from an intron of the *SPRY4* gene (48). In contrast to *HOTAIR*, very little is known about the cellular function of *SPRY4-IT1*, which was reported to be differentially expressed in melanoma by Khaitan *et al* (48). The authors utilised a microarray approach to determine differences in lncRNA expression between the stage III melanoma cell line, WM1552C, and control melanocytes. Alongside this, patient

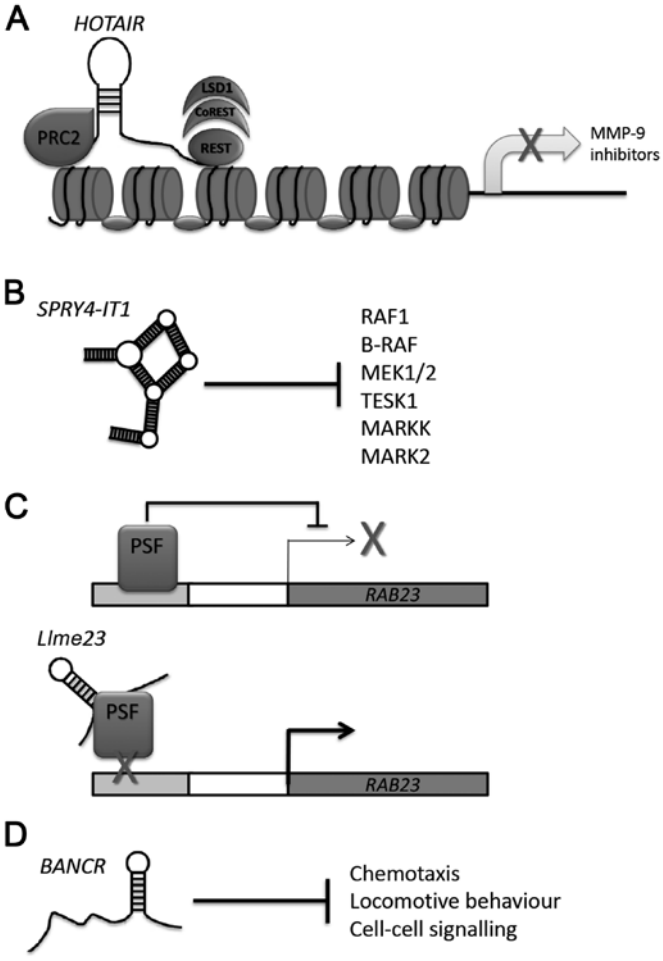

Figure 1. Putative functional mechanisms for melanoma-associated long non-coding RNAs (lncRNAs). (A) *HOTAIR* is likely to be downregulating MMP-9 expression by functioning *in trans* to recruit chromatin modification complexes to upstream regulatory regions. (B) The cellular mechanisms underpinning *SPRY4-IT1* function remain to be elucidated; however, it is likely that MAPK pathways will be targeted given the regulatory role of SPRY4 in these signalling networks. (C) *Llme23* physically interacts with protein-associated splicing factor (PSF) leading to its disassociation from upstream regulatory elements in the RAB23 locus, thereby leading to overexpression of *RAB23* and oncogenesis. (D) Depletion of *BRAF*-activated non-coding RNA (*BANCR*) resulted in the differential expression of 88 genes that were enriched for transcripts with gene ontology terms associated with metastasis.

samples isolated from stage I-IV disease were also assessed and results aligned with cell line data to identify lncRNA transcripts that were differentially expressed in both experimental systems. This careful approach, validated by NGS, identified four lncRNA transcripts, three of which were disregarded due to their location in the 3' UTR of protein-coding genes and concerns over the technical difficulties this would present for downstream analysis, the remaining transcript, which also displayed the greatest differential expression (~12-fold), was *SPRY4-IT1* (48).

In addition to increased expression in melanoma, the *SPRY4-IT1* lncRNA is also of interest due to its predicted secondary structure. Analysis of *SPRY4-IT1* using RNAfold and RNAstructure (49,50) revealed several putative regulatory motifs, including three nested helices and two 'pyknons' motifs (48). The presence of pyknons (non-random, genome-wide motifs) is particularly intriguing as it has been suggested that these elements play a role in post-transcriptional gene silencing (51), indicating that *SPRY4-IT1* may directly affect gene expression, something yet to be confirmed experimentally. Depletion of *SPRY4-IT1* in A375 cells using siRNA and scramble controls resulted in decreased metabolic viability and increased apoptosis via MTT and Annexin V assay, respectively. Moreover, *SPRY4-IT1*-depleted A375 cells exhibited significant reduction in both cell invasion and cell motility when compared with scramble siRNA-transfected control. Importantly, each of these phenotypic changes can be ascribed to reduced *SPRY4-IT1*, as knockdown of the lncRNA did not alter the expression of its 'host' gene *SPRY4*. Furthermore, overexpression of *SPRY4-IT1* in LOX-IMV1, a metastatic melanoma cell line shown to express only low-levels of *SPRY4-IT1*, significantly increased cell motility in a wound healing assay (48).

A defined cellular function for *SPRY4-IT1* remains elusive, however, it has recently been linked with the aetiology of preeclampsia and oesophageal squamous cell carcinoma (52,53). One possible clue may reside in the geographical location of *SPRY4-IT1* within the intronic sequence of the *SRYY4* gene, a property that may indicate a biological function that is linked to that of the 'host' gene (54). *SPRY4* is a member of the Sprouty family of Ras/ERK inhibitors proteins that prevent the formation of active GTP-RAS and are therefore considered to be putative tumour suppressors (55,56). Clearly, *SPRY4-IT1* is not operating as a tumour suppressor in melanoma; however, it may manipulate similar intrinsic cellular pathways, such as Ras/ERK (Fig. 1B). Future experiments, such as investigating the effect of Ras/ERK inhibitors on increased cellular motility following *SPRY4-IT1* overexpression will likely prove informative and are of clinical importance given emerging targeted therapies for melanoma that focus on MAPK-signalling (57).

Llme23. Thus far, we have considered lncRNAs identified due to their differential expression in metastatic melanoma. The lncRNA *Llme23* differs in this respect as while it appears to be exclusively expressed in melanoma, it was first described using assays designed to identify functional lncRNA partners to the polypyrimidine tract-binding protein-associated splicing factor (PSF) (58). The PSF protein is a somewhat intriguing molecule that was originally thought to operate as a splicing factor, having been identified in spliceosomal extracts (59). However, subsequent analysis has revealed a tumour-suppressor function for this protein. Study carried out in mouse models demonstrate that PSF is able to regulate the transcriptional activity in multiple proto-oncogenes via its DNA-binding domain (DBD), which interacts with the regulatory regions of these targets, repressing their expression (60).

Fascinatingly, this tumour-suppressor function is eradicated by the binding of a mouse retrotransposon lncRNA, *VL30-1*, to the RNA-binding domain motifs present in PSF. Moreover, retroviral transmission of *VL30-1* to human melanoma cells promoted metastatic progression in immunocompromised mice, suggesting a possible role for PSF in the aetiology of metastatic melanoma (61,62). While attention-grabbing, the absence of a human homologue of the mouse *VL30-1* lncRNA raised doubts regarding the existence of a similar intrinsic mechanism in humans. However, recent study by

Wu *et al* describes a human lncRNA that interacts directly with PSF in order to drive melanoma tumour formation (58). Here an RNA-SELEX approach was utilised to enrich for human RNAs that bind PSF and via subsequent cDNA library construction and electromobility gel-shift assays, a 1,600 nt lncRNA, termed *Llme23*, was identified that binds directly to PSF. These *in vitro* studies were also confirmed *in vivo* by RNA-immunoprecipitations and downstream functional experiments confirmed that *Llme23* binding to PSF inactivated PSF-mediated repression of *RAB23*, confirming that the human *Llme23* lncRNA also inhibits PSF's tumour-suppressor function (Fig. 1C), as is the case with the murine lncRNA *VL30-1* and PSF in the mouse model system (61).

How then is Llme23 impacting on melanoma aetiology? Data investigating the effects of Llme23 overexpression and depletion is more limited than for *HOTAIR* and *SPRY4-IT1*, however, Llme23-depleted YUSAC cells displayed a significant decrease in their ability to form colonies in soft-agar and most significantly, these same cells displayed ~75% decrease in tumour volume at day 38 post-injection into nude mice (58). Taking into consideration the emerging literature around PSF and cancer (63,64), it will be extremely interesting to observe the delineation of *Llme23* function in the literature, which to date is restricted to the article discussed here.

BANCR. As discussed above, derailment of the Ras/ERK MAPK signalling cascade is extremely common in metastatic melanoma. Our understanding of the molecular events that underpin such deregulation took a conceptual leap forward in 2002 with the discovery of mutations in the v-RAF murine oncogene homologue B (BRAF) proto-oncogene (65). The significance of this finding is linked to the prevalence of BRAF mutations in cutaneous melanoma, with >50% of tumours harbouring mutations in BRAF and crucially >90% of these cases possessing the same $BRAF^{V600E}$ substitution (66,67). This realisation resulted in a focused effort to develop $BRAF^{V600E}$-specific inhibitors and has resulted in the development and release of two drugs, vemurafenib and dabrafenib, which received approval by the FDA for the treatment of $BRAF^{V600E}$ mutant melanoma in 2011 and 2013, respectively. Unfortunately, despite excellent response rates of ~50%, the vast majority of patients treated with these drugs relapse and progress to chemoresistant disease that is generally fatal. A number of mechanistic explanations have been identified to explain this relapse, with the majority linked to the reactivation of aberrant MARK-signalling, although other pathways (insulin growth factor receptor and platelet-derived growth factor receptor) have also been implicated (68). Clearly, there is an urgent need to better understand how oncogenic BRAF interacts with the cellular machinery to impact disease.

Flockhart *et al* set out with just this goal in mind as outlined in their recent article, which describes the use of RNA-seq on primary human melanocytes transduced with lentivirus expressing either $BRAF^{V600E}$ or red fluorescent protein control in order to specifically identify differentially expressed lncRNAs (69). This approach represents an important shift away from other studies, which have generally focused on lncRNAs that are differentially expressed in cancer, without first addressing the cause of the observed differential expression and whether it is driven by oncogenic events or an artefact of tumour heterogeneity and genomic instability.

In addition to determining global transcriptomic changes in primary melanocytes expressing $BRAF^{V600E}$, RNA-seq data were granted more clinical credence by concurrently analysing $BRAF^{V600E}$-positive melanoma tissue samples. Indeed, results were processed through a rigorous study-flow that utilised cross-referencing with a publically available melanoma RNA-seq data set (70) and interrogation of the ENCODE RNA-seq data in order to validate that identified transcripts are actively transcribed in melanocytes. Finally, transcripts of interest were analysed using a previously described coding potential calculator (CPC) algorithm that discriminates coding from non-coding transcripts (71). This combinatorial approach identified a novel lncRNA that is overexpressed in $BRAF^{V600E}$-positive melanocytes and melanoma, which the authors termed *BRAF*-activated non-coding RNA (*BANCR*) (69).

Functional experiments depleting *BANCR* in melanoma cells did not result in decreased viability and proliferation. However, consistent with *HOTAIR*, *SPRY4-IT1* and *Llme23*, depletion of *BANCR* did significantly reduce melanoma cell motility. Gene expression profiling carried out on Colo829 $BRAF^{V600E}$-depleted melanoma cells using a cDNA microarray revealed that 88 genes were differentially expressed compared with control and significantly genes involved in cell motility were overrepresented among this number (Fig. 1D) (69). In order to gain some functional insight into how *BANCR* might be regulating cell migration, targets that displayed altered gene expression in *BANCR*-depleted cells and are associated with cell motility were investigated further. Interestingly, the chemokine CXCL11, the expression of which appears to be positively regulated by *BANCR*, was able to rescue the reduced cell motility phenotype observed in *BANCR*-depleted melanoma cells. These data suggest a scenario where the $BRAF^{V600E}$ mutation induces overexpression of *BANCR*, which in turn then positively regulates expression of CXCL11 in order to promote cell migration. This observation may be of clinical importance as activation of the chemokine receptor, CXCR3, has been linked to lymph node metastasis in melanoma (72).

3. Conclusions

Over the past decade, the explosion of research on non-coding transcripts has left us in little doubt that our genomes exhibit great transcriptional complexity. However, our understanding of how this complexity links to function, if at all, remains far less assured. Clearly, lncRNAs are key players in cancer progression and exhibit huge potential as biomarkers and novel therapeutic targets for treatment. Perhaps the biggest challenge facing researchers is determining which of the many thousands of transcripts are truly functional. Classical single 'gene' experimental analysis remains central to this process, but with improved read-length technologies for NGS on the horizon it is likely that such studies will benefit greatly from improved functional annotation. A second challenge relates to our understanding of how lncRNA structure can be analysed and used to predict function and accurately identify target genes via bioinformatics. Certainly there is a long and

somewhat uncharted road ahead. Flockhart *et al* (69), identified an additional 38 annotated lncRNA transcripts, in addition to *BANCR*, that are regulated by $BRAF^{V600E}$ and expressed in melanoma and similar reservoirs likely remain untapped from other melanoma RNA-seq data sets. Furthermore, the array of regulatory mechanisms that impact on non-coding RNA function continues to grow. Methyl-6-adenosine modification of RNA transcripts was recently shown to be a reversible event that is thought to regulate mRNA and lncRNA stability and has ushered yet another area of gene expression research, RNA epigenetics (73). While the challenges are numerous, the rewards are significant, characterisation of functional lncRNAs and their modes of action will provide exciting opportunities to augment and improve melanoma diagnosis, prognostic monitoring and targeted therapies.

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