RESEARCH NOTE

Study of bantam miRNA expression in brain tumour resulted due to loss of polarity modules in *Drosophila melanogaster*

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Abstract

Disturbance of delicate concordance between stem cell proliferation, specification and differentiation during brain development leads to several neural disorders including tumours. Accumulating evidences have demonstrated involvement of short noncoding microRNAs (miRNAs) in governing several biological as well as pathological processes, including tumourigenesis across various species. *Drosophila* bantam miRNA, known to regulate critical physiological functions is reported to have elevated expression in ovarian tumour. Here, we provide an update on the expression of bantam miRNA in *Drosophila* brain tumour background resulting due to loss of well characterized polarity proteins, Brat, Lgl and Scrib. Since, both miRNA TaqMan assay and bantam sensor assay showed elevated expression of bantam in brain tumour background, it clearly reflects presence of an antagonistic relationship between polarity proteins and bantam miRNA indicating of its involvement in tumour progression.

[Banerjee A. and Roy J. K. 2017 Study of bantam miRNA expression in brain tumour resulted due to loss of polarity modules in *Drosophila melanogaster*. *J. Genet*. **96**, 365–369]

Introduction

At cellular level, tumourigenesis involves a series of events leading to acquisition of morphological and physiological changes of the tissue/organ. In humans, evidences have suggested that irregularities in expression of endogenous, 22 nucleotides long-noncoding microRNA (miRNA/s) directly or indirectly involved in different disorders including cancer. Many miRNAs not only behave as oncogenes and tumour-suppressors but also regulate key signalling pathways critical for normal cell cycle progression (Jansson and Lund 2012). For centuries, *Drosophila*, whose genome shares striking similarities with the mammalian genome, played a pioneer role in understanding the complex molecular pathways of mammalian system (Pandey and Nichols 2011). *Drosophila* has several tumour-suppressor genes which show structural and functional homology with their mammalian counterpart. Among them, the polarity modulators, namely, *brain tumour* (*brat*), *scribble* (*scrib*) and *lethal (2) giant larvae* (*lgl*) are three well-studied tumour-suppressors in *Drosophila* (Albertson and Doe 2003; Humbert *et al.* 2008). Mutation in either of these genes lead to neoplasia or over proliferation of neuroblasts (neural stem cells) along with tumours in different tissues and other associated anomalies (Wirtz-Peitz and Knoblich 2006; Bowman *et al.* 2008). Loss of function of Brat homologue, Mei-P26, a critical regulator of germline stem cell division leads to a higher expression of bantam in ovary (Neumuller *et al.* 2008). Bantam, is a well-characterized miRNA in *Drosophila* which is a positive regulator of tissue growth (Hipfner *et al.* 2002). Here, we report an elevated expression of bantam miRNA in the mutant background of three tumour-suppressor, *brat*, *scrib* and *lgl*.

Materials and methods

Drosophila stocks

Unless otherwise stated, wild type (*Oregon* R^+) and mutant flies were reared on standard yeast–cornmeal–agar medium and all experiments were performed at 24 ± 1°C. The following fly strains were used in this study: $brat^{11}$ and $brat^{14}$ (D. J. Frank, Washington, USA), $bantam^{12}$ (null allele) and $bantam^{20}$ (hypomorphic allele) (P. Jin, Atlanta,

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Keywords. brain; microRNA; tumour; cell polarity; neuroblast; bantam.

USA), *bantam sensor* (G. Halder, University of Texas, USA). All the other stocks mentioned in the manuscript were obtained from Bloomington Stock Centre.

Immunofluoresence

Late third instar larval brain were dissected in 1× phosphate-buffered saline, fixed in 4% paraformaldehyde for 20 min at room temperature, and preceded as previously described (Banerjee and Roy 2017). DNA was visualized using DAPI (1 μg/mL) (Sigma-Aldrich, St Louis, USA).

Microscopy and image analysis

All fluorescent images were captured using LSM 510 Meta confocal microscope with picture size 1024 × 1024 pixel and processed in Photoshop 7 (Adobe, San Jose, USA). For measuring the intensity of fluorescence, projection of image was obtained from selected z sections and measured using LSM 510 Meta software.

MiRNA TaqMan assay

For quantification of bantam miRNA expression in the desired genetic background, we used ABI (Applied Biosystem, Foster City, USA) miRNA TaqMan assay. Late third instar larval brain was dissected in 1× PBS and kept in Tri Reagent (Sigma-Aldrich). RNA from 50 wandering third instar larval brains was isolated using Tri Reagent as per the manufacturer's instruction followed by DNase I (Thermo Scientific, Waltham, USA), treatment to rule out DNA contamination. Reagents of TaqMan miRNA assay were used to prepare cDNA from 10-ng RNA. In a Micro Amp optical 96-well plate, real-time PCR reactions were performed using 7500 fast real-time PCR system (ABI) in triplicate with specific forward and reverse primers (ABI) following manufacturer's protocol. For normalization, 2S RNA was used that showed no change in different genetic backgrounds.

Results

Since the level of bantam expresses in brain (Brennecke *et al.* 2003) were reported to be elevated in ovarian tumour caused in *mei-P26* mutants (Neumuller *et al.* 2008), we were inquisitive to investigate expression of bantam in brain tumour in *brat* mutant background. To assess the levels of mature bantam miRNA, we performed miRNA TaqMan assay with 2S RNA as an internal control using primers for bantam and 2S RNA (Applied Biosystems) in tumours brain of *brat* mutants. Results of TaqMan assay showed elevated level of bantam in the $brat^{11/14}$ *trans*-heterozygotes compared to the wild type (figure 1a) indicating antagonistic relationship between *brat* and *bantam*.

During asymmetric division of neuroblasts, proper segregation of apical and basal fate determining factors are a crucial event which establishes polarity and maintains cell fate of the progenitor cells after division (Homem and Knoblich 2012). *Lgl*, *Scrib* and *Brat* are important candidates involved in asymmetric division of neuroblasts (Albertson and Doe 2003). Loss of function in any of these genes lead to an inappropriate segregation of cell fate determinants which cause formation of supernumerary self-renewing daughter cells instead of differentiated cells resulting in an uncontrolled proliferation, consequently giving rise to a tumour (Wirtz-Peitz and Knoblich 2006; Bowman *et al.* 2008). Thus, we repeated our experiment in *lgl* and *scrib* mutant background, to study whether change in bantam expression was *brat* mutant specific or due to loss of polarity in dividing neuroblast. In lgl^{4} and $scrib^{M101968}$ loss of function mutants, we observed significant elevation in bantam level as compared to control individuals (figure 1a). To consolidate our findings, we induced expression of *bantam sensor* in $brat^{11/14}$ trans-heterozygote background and examined the expression of bantam (figure 1, b&b′). *Bantam sensor* flies have a transgene with a green fluorescent protein (GFP) protein-coding region under the tubulin promoter and its 3′UTR consisting of two perfect bantam miRNA-binding sites. The cells expressing bantam miRNA are GFP negative, as bantam interacts with RNA induced silencing complex (RISC) to inhibit GFP translation by binding to the 3′UTR region of this sensor transcript. The cell that does not have bantam miRNA expression has GFP due to the absence of a translational blockade (Brennecke *et al.* 2003). Hence, the expression of this GFP coding transgene is opposite to bantam activity in a particular cell type or tissue. Quantitative estimation of GFP fluorescence intensity in third instar larval brain, revealed that the mean intensity of GFP was significantly higher in wild type as compared to the *brat* mutants (figure 1c) indicating greater number of cells expressing bantam in mutant brain. Since, it was reported that *bantam* mutant individuals have smaller tissue size including brain compared to normal (Brennecke *et al.* 2003) and from our findings that it has elevated expression in tumourous background, we brought *bantam* null and hypomorphic allele in *brat trans*-heterozygote background, to confirm whether bringing down bantam level could rescue tumourous phenotype. Surprisingly, it could not rescue the tumourous phenotype due to *brat* mutation instead it led to an early larval lethality compared to *brat trans*-heterozygote. Above results indicate that higher expression of bantam in tumourous background could possibly be a consequence of brain tumour resulting from *brat*, *scrib* and *lgl* mutation.

Discussion

In *Drosophila*, stem cells are found in testis, ovary, midgut, Malpighian tubules, haemolymph and the neuroblast lineage of brain (Kohlmaier and Edgar 2008). In brain, the

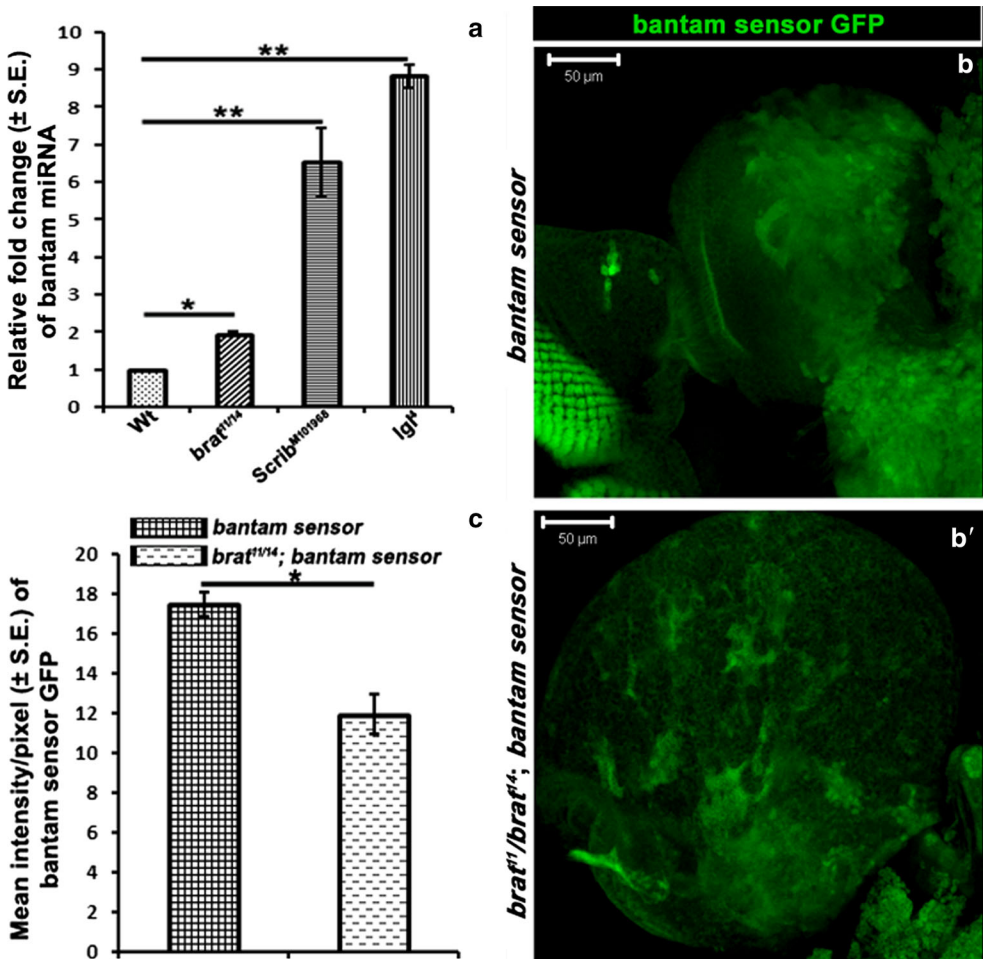

Figure 1. Bantam is upregulated in tumorous background. (a) miRNA TaqMan assay was performed with total RNA from third instar larval brain of different genotypes, namely, wild type (117 ± 1 h), $brat^{11/14}$ (192 ± 3 h) and $scrib^{M101968}$ (192 ± 3 h), lgl^4 (192 ± 3 h) to compare the level of mature bantam miRNA. The Y-axis shows relative fold change of bantam expression in different genotypes mentioned along the X-axis. (b-b′) Representative optical projection showing enrichment of bantam sensor GFP in mutant background (b′) compared to bantam sensor used as control (b). (c) Graphical representation showing mean fluorescence intensity of bantam sensor GFP and third instar larval brain of mentioned genotypes. Statistical significance was calculated using one way ANOVA (a) and student's *t*-test (c). All the quantification data show mean ± S.E. $^{**}P < 0.001$ and $^{*}P < 0.05$.

neuroblasts like other stem cells undergo asymmetric division to produce a larger cell (neuroblast) which retains the regeneration capability and a smaller ganglion mother cell (GMC). GMCs undergo one more round of division to generate target neurons or glia. Brat along with other associate proteins, Miranda and Prospero, form the cell fate determination complex to mediate the asymmetric division of the neuroblast. Apical proteins, Par-3 and Par-6, atypical protein kinase C (aPKC) helps in a basal localization of cell fate determination complex which after asymmetric division destined to form the GMC. Phosphorylation of Lgl by aPKC on apical side confines the Lgl activity to the basolateral domain of cell (Humbert *et al.* 2008; Homem and Knoblich 2012). Further, disc large (Dlg) serves as an additional regulator for proper localization of Scrib and Lgl in neuroblasts. Lgl and Scribble in turn functions for basal localization of cell fate determinants in metaphase neuroblast but not in telophase neuroblast (Albertson and Doe 2003). Brat along with Prospero after segregating in GMCs promotes cell cycle exit and ultimately leads to the formation of ganglion cells (Wirtz-Peitz and Knoblich 2006; Bowman *et al.* 2008; Homem and Knoblich 2012). Mutation of *lgl* and *scrib*, perturbed the machinery regulating the neuroblast self-renewal, generating excesses number of neuroblasts and their progeny. The neuronal progenitor cells in the *brat* loss of function mutants fail to escape the cell cycle leading to exponential proliferation of cells causing brain tumour (Bowman *et al.* 2008).

Bantam miRNA was first reported in *Drosophila* during a gain-of-function screen, it governs tissue growth by regulating cell proliferation and apoptosis (Hipfner

et al. 2002). In *Drosophila*, bantam plays a wide range of functions which includes maintenance of germline and intestinal stem cell (Yang *et al.* 2009; Huang *et al.* 2014), polyQ and tau-mediated neurodegenaration and various others (Bilen *et al.* 2006). Further, bantam was reported to be involved in Hippo and EGFR mediated tumourigenesis (Nolo *et al.* 2006; Herranz *et al.* 2008). All these facts made us curious to explore the expression of bantam in *Drosophila* brain tumour. We carried out miRNA TaqMan assay that revealed an increased expression of mature bantam miRNA in $brat^{11/14}$, $scrib^{M101968}$ and lgl^{4} brain tumour backgrounds. Decrease in bantam sensor GFP expression in the tumourous brain of $brat^{11/14}$ transhetrozygous further corroborates our findings from TaqMan assay. Results from our preliminary study indicates bantam miRNA could probably be playing some role in brain tumours caused due to mutation of molecular signatures involved in distribution of cell fate determinants during asymmetric division of neuroblasts. However, inability to rescue $brat^{11/14}$ tumorous phenotype by bringing down *bantam* level using *bantam* null and hypomorphic allele could be because of either increased number of neuroblast-like cells in *brat* mutant plausibly leads to a higher expression of bantam in tumorous background or since bantam expresses in different tissues in spatiotemporal manner during *Drosophila* development (Brennecke *et al.* 2003), its null and hypomorphic allele will affect its function in different tissues including brain leading to early larval lethality. Recently, in a parallel study, Cohen and Weng (2015) showed higher expression of brat, prospero and numb transcripts in *bantam* mutant neuroblasts.

In *Drosophila*, bantam and its target SOCS36E (human ortholog of SOCS5) are reported to be cooperating factors in EGFR mediated tumourigenesis (Herranz *et al.* 2012). Further, bantam is known to be an important target of the highly conserved Hippo tumour-suppressor pathway (Nolo *et al.* 2006). Accumulating evidences and our study suggest bantam to be an important candidate in *Drosophila* tumourigenesis. Intriguingly, Scrib, Lgl and Brat are involved in basal segregation and formation of cell fate determining complex and mutation of these polarity determining candidates leads to transformation of certain neuroblast population. Thus, future experiments exploring function of bantam during asymmetric division of these neuroblasts and its relationship with molecular machineries involved in brain tumour will help us to gain new insights about role of bantam during *Drosophila* neurogenesis and brain tumour progression. Mutation of *scrib* and *lgl* results in epithelial tumours (Humbert *et al.* 2008). It will be of interest to study expression and function of bantam in these epithelial tumours. Even though, bantam is not conserved in mammals, its target and regulators are highly conserved along the evolutionary tree and thus, further studies possibly could lead to find novel targets involved in vertebrate brain tumours.

Acknowledgements

We thank the fly community for generously providing fly stocks. We duly acknowledge Banaras Hindu University for providing the National facility for Laser Scanning Confocal microscopy and Invitrogen Bio Services, Gurgaon, India and Interdisciplinary School of Life Sciences, Banaras Hindu University for extending thermocycler facility.

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Received 9 July 2016; accepted 13 July 2016
Unedited version published online: 15 July 2016
Final version published online: 19 June 2017

Corresponding editor: L. S. SHASHIDHARA