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Mutations in a Gene Encoding a Midbody Protein in Binucleated Reed-Sternberg Cells of Hodgkin Lymphoma

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Abstract

Classical Hodgkin lymphoma (cHL) is a cancer in which malignant "Reed-Sternberg" cells comprise just a fraction of the bulk of the tumor and are characteristically binucleated. We recently identified a novel gene, *KLHDC8B*, which appears responsible for some familial cases of cHL. *KLHDC8B* encodes a midbody kelch protein expressed during cytokinesis. Deficiency of *KLHDC8B* leads to binucleated cells, implicating its involvement in Reed-Sternberg cell formation. Interestingly, other cancer genes, such as *BRCA1* and *BRCA2*, also encode proteins locating to the midbody during cytokinesis, even though their participation in other pathways has received greater attention. Midbody components may be an overlooked source of tumor suppressor genes.

Keywords

Hodgkin lymphoma; cytokinesis; midbody; *KLHDC8B*; Kelch; *Keap1*; *BRCA1*; *BRCA2*; *BARD1*

Classical Hodgkin lymphoma

Classical Hodgkin lymphoma (cHL) is a cancer arising in B-lymphocytes of the germinal center of lymph nodes[1]. Unlike other malignancies, it demonstrates a bimodal age distribution with peak incidence both in early and late adult years. Along with African Burkitt lymphoma, nasopharyngeal carcinoma in Southeast Asia, and lymphoproliferative disease found among post-transplant immunosuppressed patients, cHL is associated with Epstein-Barr virus[2]. Another distinguishing feature is that the overwhelming bulk of the tumor is comprised of benign reactive inflammatory cells; malignant cells are few and consist of mononucleated "Hodgkin cells" and pathognomonic bi- or multi-nucleated "Reed-Sternberg" cells.

The familial risk for cHL ranks amongst the highest for all types of cancer[3], yet except for HLA associations[4] and its occurrence in rare, pervasive immunodeficiency disorders[5], genes

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underlying predisposition to cHL have remained largely undefined. We recently identified a novel gene that is likely to be responsible for some familial cases of Hodgkin's lymphoma and that may shed light on molecular mechanisms contributing to this and other forms of cancer.

Cloning and Characterization of KLHDC8B

In a recently published paper[6], we ascertained a family where several individuals inheriting a constitutional, balanced translocation between chromosome 2 and 3 developed cHL. We molecularly cloned the breakpoints and determined that the translocation disrupts the previously uncharacterized gene, *KLHDC8B*, located on chromosome 3p21.31, by deleting its upstream regulatory elements and first exon and fusing a portion of its 5′-UTR to an intergenic region on chromosome 2q11.2, thereby abrogating its transcription. Additionally, we found that a rare SNP at a highly phylogenetically conserved position within the 5′-UTR reduced translational expression of *KLHDC8B* and was associated with and linked to cHL in three other families. Several people with this SNP also developed lung cancer instead of, or in addition to, cHL. Moreover, in one of three sporadically occurring cHL tumors, where genetic markers were informative, purified Reed-Sternberg cells demonstrated loss of heterozygosity (LOH) for *KLHDC8B*.

There is evidence that this region of chromosome 3 contributes to other forms of cancer. It is a site of recurrent cytogenetic abnormality and LOH in lymphoma[7] as well as breast[8] and lung and other types of cancer[9], which share a genetic epidemiologic association with cHL[10], and there are linkage data[11], LOH analysis[12], and chromosome transfer experiments[13] implicating its involvement in nasopharyngeal carcinoma, another of the EBV-associated malignancies.

Genetics notwithstanding, it is the apparent function of KLHDC8B which may offer a clearer picture of early pathomolecular processes leading to cHL.

KLHDC8B is one of 71 known or predicted "kelch" family genes residing in the human genome[14]. The kelch motif comprises an approximately 50 amino acid residue repeated sequence[15] first detected in a *Drosophila* protein serving as a component of "ring canals"[16], which form intercytoplasmic bridges that connect primordial germ cells and that arise through a process of incomplete cytokinesis following cell division. Other kelch family members participate in diverse biochemical activities and consist of five, six, or seven repeated kelch domains. Most contain additional features, the most common being a "BTB/POZ" domain. *KLHDC8B* distinguishes itself by being one of just two human genes containing seven kelch repeats but lacking other motifs.

The kelch domain is recognized for its ability to bind actin and participate in protein-protein interactions. Kelch proteins adopt a beta-propeller structure. The crystal structure[17] for only one seven-repeat kelch-only protein (a fungal galactose oxidase) has been resolved; we have used it as the basis for modeling[18] the predicted structure of KLHDC8B (Fig. 1). Note that the seventh kelch domain is split between amino and carboxyl termini, whereas for those containing fewer numbers of repeats, each kelch domain is contiguously coded within the gene.

Although *KLHDC8B* is widely expressed, per gene expression atlases, germinal center B lymphocytes, the malignant cell of origin in cHL, do so most abundantly[19], further implicating its involvement in lymphomagenesis. In order to decipher the function of KLHDC8B, we generated antibodies and performed indirect immunofluorescent staining[6] of HeLa cells, which, although not necessarily relevant to lymphoma, are flat and have an abundance of cytoplasm, affording easy visualization (Fig. 2). KLHDC8B is expressed only in mitotic cells, where it locates to the midbody—a small intracellular structure that serves as the last point of contact between dividing cells before they undergo separation through the process of cytokinesis[20]. Moreover, quantitative RT-PCR and western blots performed on synchronized cell populations indicate that *KLHDC8B* is transcribed during S-phase and confirms that the protein is predominantly present, and therefore presumably translated, during cytokinesis, followed by its rapid degradation[6]. Based on its timing and location of expression, KLHDC8B appears to participate in cytokinesis.

KLHDC8B's mitotic expression also suggests why the 5′-UTR SNP that we observed to be associated with and linked to cHL in additional families may so profoundly disrupt translation. The SNP, a C to T base substitution, is located in a polycytidine tract in a run of several adjacent polycytidine repeats. Clustered polycytidine repeats can function as an internal ribosome entry site (IRES)[21]. It turns out that mitotically expressed genes rely on CAP-independent translation of mRNA and therefore require an IRES, even for polypeptide synthesis commencing from an ATG located at the 5′ end of the transcript[21, 22]. Thus, the SNP likely disrupts an IRES required for translation of KLHDC8B specifically during mitosis.

We employed RNAi to knockdown expression of KLHDC8B in HeLa cells and found that reduction of KLHDC8B increased the proportion of binucleated cells[6]. We corroborated this observation by manufacturing a dominant negative form of KLHDC8B (M. Krem, unpublished results) that also creates binucleated cells, by interrupting the proper sequence of cytokinesis (Fig. 3). This observation is significant with respect to cHL, because the tumor's signature binucleated Reed-Sternberg cell forms as a consequence of defective cytokinesis, rather than through cell fusion[23–26]. Therefore, haploinsufficiency of KLHDC8B may promote Reed-Sternberg cell formation.

How a binucleated cell, whose further division may be sluggish at best, may contribute to malignancy is uncertain. One possibility is that it is indicative of an underlying cytokinesis defect that may also result in aneuploidy in mononuclear cells. Not surprisingly, some of the most conspicuous mutations in cHL are chromosomal aberrations, and chromosomal instability is strongly implicated in Reed-Sternberg cell formation and cHL pathogenesis. Case series show strong evidence of chromosomal instability and chromosomal aberrations in most cases of HD[27, 28]; there is a particularly heavy frequency of tetraploidy or near-tetraploidy[29, 30]. It has been speculated that Reed-Sternberg cells or their immediate precursors are derived from a karyotypically aberrant lineage[1]. The uniquely high frequencies of tetraploidy and near-tetraploidy correlate closely with multinucleation; the accumulation of extra chromosome sets would be simply explained by a defective cytokinesis model.

Of note, an affected woman in the translocation family gave birth at an early age to a child with trisomy 21 Down syndrome[6]. One speculative interpretation is that KLHDC8B, as with *Drosophila* Kelch, is active during gametogenesis. If so, another speculative hypothesis—based on an observation of an abundance of twins in the translocation family and an association between twinning and cHL in general[31]—is that loss of KLHDC8B may lead to twinning through persistent cytoplasmic bridges between oocytes derived from common progenitors. In a separate paper[32], we have explored that possibility, by genetically determining if one twin from a pair in this family might be derived from a polar body.

Kelch and Other Proteins Involved with Cytokinesis

Several other kelch proteins are involved in cytokinesis. Tea1p, containing kelch repeats along with coiled-coil domains, interacts with microtubules to correctly position the cell division plane in fission yeast[33]. Similarly, Kel1p and Kel2p, also containing kelch repeats and coiled-coil domains, act in concert with the actin cytoskeleton to help localize the neck separating mother and daughter cells in budding yeast[34]. Nd1, which contains kelch repeats and a BTB/POZ domain, colocalizes with actin, and its over-expression retards cytokinesis by interfering with reorganization of the cytoskeleton as mitosis progresses[35]. Another BTB/POZ-kelch protein, Keap1, has additionally been isolated as a component of the midbody[36]. Two more BTB/POZ-kelch proteins, KLHL9 and KLHL13, are found in the midbody[37], where they bind to the Aurora B kinase spindle checkpoint regulator and act as adapter proteins that, analogously to Keap1's interaction with Nrf2, direct ubiquitination of Aurora B by cullin 3-based E3 ligase[37]. Thus, participation in cytokinesis appears to be a major function of the kelch family of proteins.

When abscission fails, cytokinesis remains incomplete, and the cleavage furrow regresses, thus leading to the formation of binucleated cells[38]. Disruption of the expression of a diversity of proteins localizing to the midbody has been consistently shown to interrupt cytokinesis and increase binucleated cell formation, as demonstrated by RNA interference-mediated knockdown of kelch proteins Keap1[36], KLH9[37], and KLH13[37]; the kinesin motor proteins MKLP1[39] and CHO1[40]; the endocytic adapter protein ARH[41]; the filament protein Tektin2[42]; the product of the tumor suppressor gene BRCA2[43]; over-expression of the vesicle membrane associated proteins syntaxin and endobrevin[44]; genetic deletion of the inner centromere protein Incenp[45]; injection of antibodies against the septin Nedd5[46]; and expression of dominant-negative forms of Aurora B[47] and the large multifunctional protein BRUCE[48]. In fact, a proteomic analysis of microdissected mammalian midbodies identified scores of proteins, the majority of which, when knocked down by RNA interference in *C. elegans*, gave rise to multinucleated cells[36].

Cancer Genes Encoding Midbody Proteins

It is interesting that some proteins (Keap1, BRCA1, BRCA2, and BARD1) involved with cancer and that are now known to locate to the midbody are thought to contribute to cancer through mechanisms completely distinct from their possible functions during cytokinesis. Could something (i.e. their function at the midbody during cytokinesis) have been overlooked, with respect to mechanisms of oncogenesis?

Keap1, a kelch protein family member containing a BTB-POZ domain, is most well studied in its capacity for sensing cellular stress. Keap1 binds the transcription factor NRF2, a master regulator of response to oxidative stress, in the cytoplasm, where, through the BTB-POZ domain, it recruits cullin E3 ubiquitin ligase to target NRF2 for ultimate proteasomal destruction[49]. However, under oxidative conditions, Keap1's ability to bind NRF2 becomes impaired, thereby both releasing it as a substrate for ubiquitination and allowing it to translocate to the nucleus, where it transcriptionally activates the stress response pathway[49, 50]. Somatic mutations in *KEAP1* have recently been described as frequent events in a number of tumors[49, 51]. At first glance, it is difficult to imagine how loss of a component needed to activate stress-induced, cytoprotective pathways could be oncogenic. One possibility is that constitutive activation of protective genes promotes growth and chemotherapeutic resistance at somewhat later stages of tumor development[49, 50]. We suggest than an alternative interpretation is that loss of Keap1, at least in the early stages of tumor formation, disrupts cytokinesis, in which case, binucleated cell formation, as observed when it is experimentally knocked down[36], could be a byproduct of a phenomenon also generating chromosomal instability. Further study of Keap1's role at the midbody in cytokinesis may be warranted.

Germline mutations of *BRCA1* and *BRCA2* cause hereditary breast and ovarian cancer and also increase risks for other types of cancer[52]. To a much lesser extent, constitutional mutations of the gene, *BARD1*, encoding BRCA1-associated RING domain-1 protein, are associated with hereditary breast cancer[53]. All three proteins have overlapping and complex, yet distinct, roles in DNA repair[54]. BRCA1 is thought to primarily function in signaling DNA damage and cell cycle regulation, whereas BRCA2 more directly participates in DNA repair[55]. Much less attention, however, has focused on the fact that BRCA1[56], BRCA2[43], and BARD1[57] all locate to the midbody during cytokinesis. Cells deficient in *BRCA2* additionally undergo a delay in cytokinesis[58]. The precedent for such a phenomenon is the rare mosaic variegated aneuploidy syndrome where heritable deficiency of the mitotic-spindle checkpoint due to loss of *BUB1B*, encoding the BubR1 kinase, generates frequent somatic aneuploidy and thereby predisposes to various types of cancer[59, 60].

Midbody Proteins, Defective Cytokinesis, and the Reed-Sternberg Cell

The mechanisms by which alterations in midbody proteins such as KLHDC8B lead to defective cytokinesis are gradually becoming elaborated. A leading hypothesis is that altered expression of spindle checkpoint genes causes centrosomal amplification, demonstrated in both mitosis[61] and oocyte meiosis[62]. Amplification of the number of centrosomes is implicated in polyploidy in both cHL cell lines and patient cases[63]. Centrosomal amplification has recently been strongly linked to chromosomal instability, as extra centrosomes promote chromosomal missegregation during cell division[64]. One might speculate that decreased expression of KLHDC8B triggers centrosomal amplification and/or aberrant spindle assembly, thus leading to defective cytokinesis and the pathognomonic Reed-Sternberg cell.

Conclusion

Whole-genome sequencing by Vogelstein and colleagues has led to the identification of twelve major pathways susceptible to tumor-inducing mutations; notably, the list did not include mitotic/spindle checkpoint proteins[65]. It is possible then that somatic or heritable mutations in genes encoding the cytokinetic machinery and that therefore contribute to faithful segregation of chromosomes may represent a significant and heretofore underappreciated category of tumor suppressors. If so, then other genes encoding components of the midbody are excellent candidate tumor suppressor genes.

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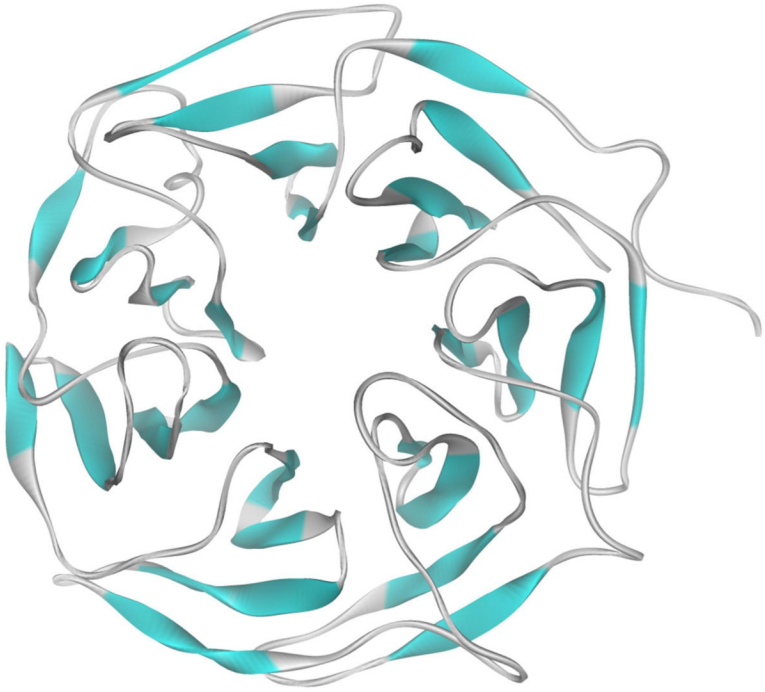

Figure 1.
Predicted structure of KLHDC8B, revealing a seven-bladed propeller structure.

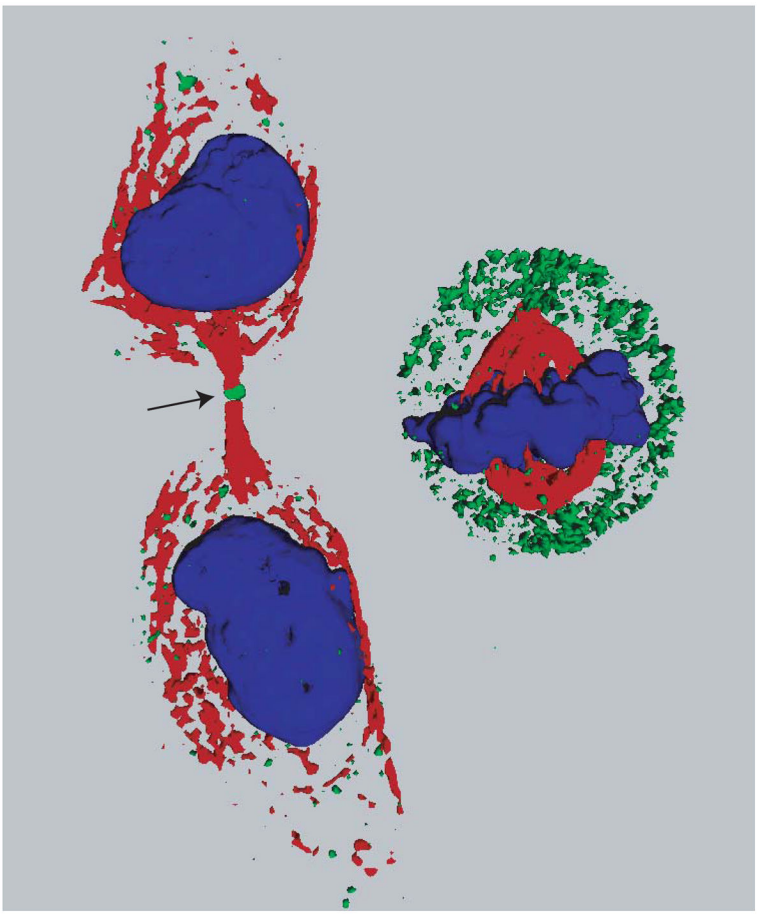

Figure 2.

Localization of KLHDC8B (green) during mitosis (right cell) and, in particular, at the midbody during cytokinesis (arrow, left cell). Counterstaining of mitotic spindle with α-tubulin (red) and nuclei using DAPI (blue). Shown is a 3D reconstruction of z-stack of confocal imaging of indirect immunofluorescence of HeLa cells.

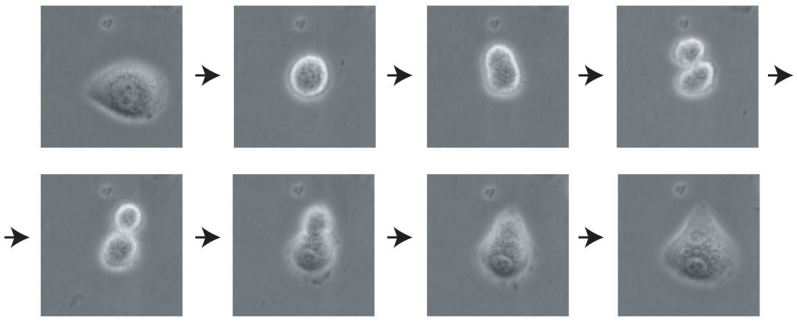

Figure 3.
Binculeated cell formation in HeLa cell expressing dominant negative KLHDC8B, time-lapse photography. Cytokinesis cannot be completed and cleavage furrow regresses.