Hyaluronan Synthases and RHAMM as Synergistic Mediators of Malignancy in B Lineage Cancers

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Abstract
Hyaluronan synthases (HASs), enzymes that synthesize hyaluronan (HA), are overexpressed, and in the case of HAS1, undergo aberrant splicing in some B lineage malignancies, including multiple myeloma (MM) and Waldenstrom's macroglobulinemia (WM). RHAMM, the receptor for HA-mediated motility, is overexpressed in malignant B lineage cells from WM, in non-Hodgkin's lymphoma (NHL), B cell chronic lymphocytic leukemia (B-CLL) and in MM. HAS1 and the HAS1 splice variants correlate with poor survival in MM. Elevated expression of the $RHAMM^{-exon4}$ splice variant also correlates strongly with reduced patient survival in MM, confirming the clinical impact of HASs and of RHAMM variant expression. We speculate that synergistic interactions between HASs, HA and RHAMM may underlie increasingly aggressive disease and the generation of chromosomal instability in MM.

Keywords
Hyaluronan, hyaluronan synthases, RHAMM, human cancer, multiple myeloma, Waldenstrom's macroglobulinemia, chromosomal instability

Introduction

Hyaluronan has complex biological effects, especially as related to cancer. Aberrant endogenous production of HA or treatment with exogenous HA *in vitro* has been shown in multiple model systems to promote cancer cell growth and malignant behavior [1]. On the other hand, treatment *in vivo* with exogenous HA can inhibit cancer growth [2;3]. In all likelihood, multiple mechanisms are involved in either stimulation or inhibition of cancer by HA. To understand the impact of HA in any given model of cancer or in cancer patients themselves, it is necessary to evaluate HA synthesis, hyaluronan synthases (HASs) and HA receptors.

HASs and B lineage malignancy

HASs have been shown to associate with malignant cell transformation [3-6] and an invasive phenotype [7]. Competition by exogenous HA inhibits tumor growth [2;3]. Of particular interest, since high dose dexamethasone is the most effective treatment for multiple myeloma, glucocorticoids induce near total suppression of HAS [8]. Our recent work shows cell-type specific expression for HAS-1 and HAS-2, while HAS-3 appears to be more ubiquitously expressed within the white blood cell types tested [9]. Human thymocytes express HAS-3, with low or undetectable expression of HAS1 and HAS 2 (unpublished). HAS is rapidly activated to synthesize HA by treatment of cells with hyaluronidase, presumably by freeing the active sites of HAS, or by other treatments that interfere with the ability of HASs to retain newly synthesized HA polymers [10]. Transitions in HAS isozyme expression, probably accompanied by the

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production of different sizes of HA molecules, may occur as cells differentiate and their behavioral requirements change. Figure 1 shows the patterns of RHAMM and HAS expression by B lineage malignancies as a function of their differentiation stages.

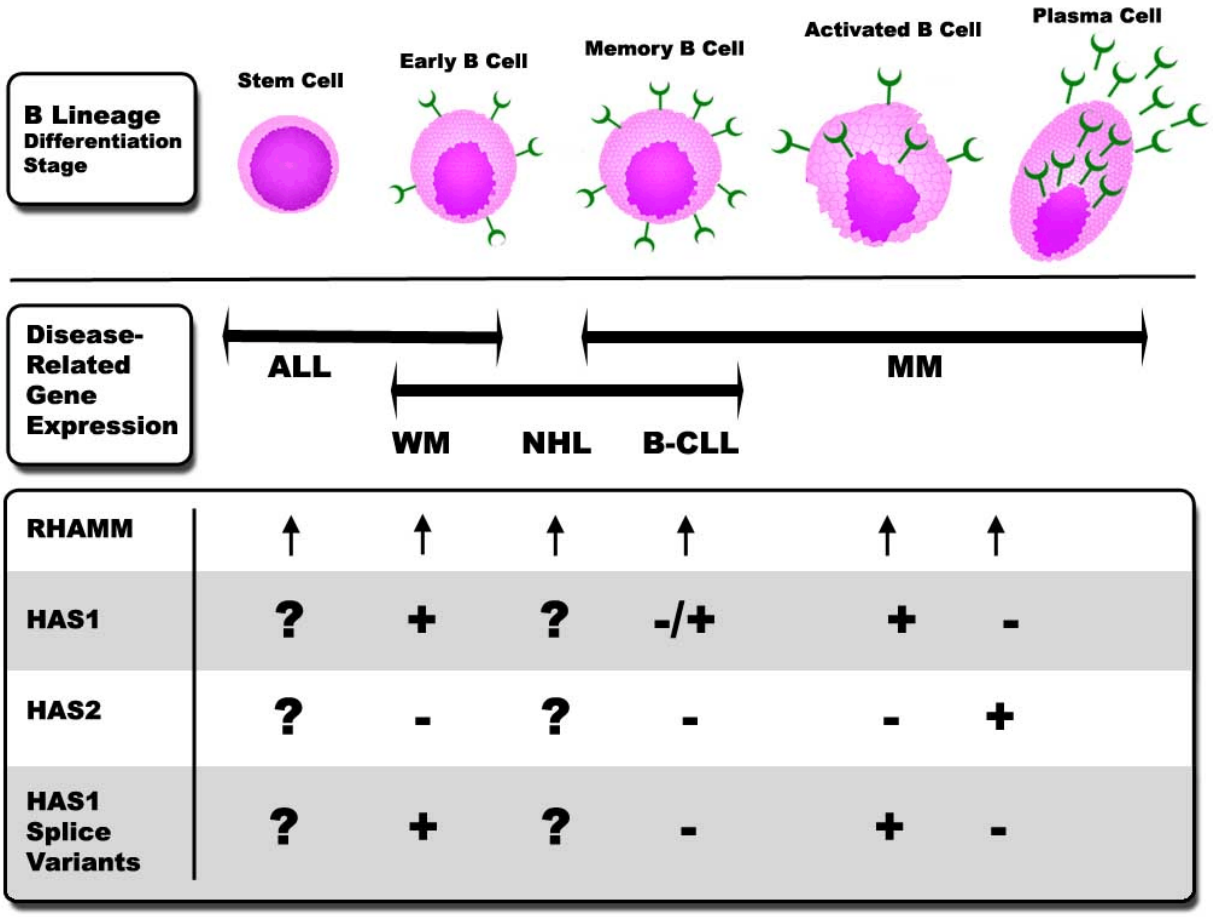

Figure 1: Expression of HASs and RHAMM in B lineage malignancies
This presents a simplified summary of cancers that involve the indicated B lineage differentiation stage (top row). B lineage malignancies involve a hierarchy of differentiation stages indicated on the figure (middle row). In some cases, the malignancy encompasses multiple differentiation stages (e.g., MM), or occurs in at least two different types of disease (e.g., B-CLL). Elevated RHAMM expression is indicated by an arrow. HAS gene expression is indicated as a +, - or ? (to indicate data that have not yet been acquired).

In MM, HAS1 and its aberrant splice variants are expressed exclusively by circulating malignant cells, while HAS2 is expressed only by bone marrow-localized/anchored malignant cells. In WM, single cell RT-PCR analysis of individual malignant B cells revealed that HAS1and the HAS1 splice variants are usually independently expressed, with frequent expression of aberrant HAS1 variants in the absence of transcript encoding the full length HAS1. (Adamia et al., in preparation). In B chronic lymphocytic leukemia (B-CLL), HAS3 is expressed, but no HAS2, HAS1 or HAS1 splice variants have as yet been detected.

In MM, only HAS1 appears to be associated with synthesis of extracellular HA, and only HAS1 and/or the HAS1 splice variants are associated with motile behavior (Table 1). The expression of HAS1 and its novel variants by motile malignant B cells suggests that HAS1 and its novel variants are involved in oncogenic processes, particularly those contributing to the spread of MM. The expression of HAS1 and HAS1variants, possibly in combination with HAS3, appears sufficient to synthesize the HA pericellular coat around MM B cells with a motile phenotype. HAS1 and/or the HAS1 splice variants are usually expressed at the time of diagnosis, become sporadically undetectable during therapy, and reemerge prior to and during relapse (Adamia et al, in preparation, [9].

Table 1: HAS1 expression correlates with synthesis of HA pericellular coats and RHAMM-dependent motility

Cell type	HAS isoenzyme expressed	HA pericellular coat	Motility
Normal B cells	HAS3	No	No
MM B cells in blood	HAS1, HAS3	Yes	Yes
MM plasma cells in bone marrow	HAS2, HAS3	No	No

HASs are all found in the blood of MM patients but, with the exception of HAS3, not in the blood of healthy donors. In MM, the presence of HAS isoenzyme variants in the blood correlates with poor survival (Adamia et al., in preparation), but to date no significant correlations have been detected for HAS isoenzyme variants expressed by bone marow-localized malignant cells. This suggests that HAS isoenzyme variants are upregulated in the blood borne components of the myeloma clone and are biologically relevant markers of circulating tumor burden. Significant correlation between poor survival and expression of HAS genes in blood borne cells are found for HAS1, HAS2, HAS1Vb and HAS1Vc. The particularly remarkable association between blood HAS1Vb and survival, and the rare detection of HAS1Vb in the bone marrow, suggests that HAS1Vb may be preferentially upregulated in circulating malignant cells. HAS1Vb is expressed by circulating B cells as identified by their phenotypic marker profiles, but is not detected in BM-localized B or plasma cells. The body of evidence accumulated so far confirms that HASs, particularly the newly identified HAS1 splice variants, appear to play important roles in disease progression in MM. In this context, Dahl et al. demonstrated that abnormally high or very low levels of HA in the serum of patients with MM correlate with dramatically reduced median survival of these patients [11]. In MM, a disease characterized by extensive overexpression of HASs and HAS1 variants, hyaluronidase is upregulated, suggesting that removal of extracellular HA may promote HAS gene expression to maintain HA homeostasis in a given tissue environment n [12]. HAS1 gene expression may promote genetic instability. This idea is supported by the observation that the circulating clonal B cells in myeloma patients are extensively DNA aneuploid with, on average, 1.07 excess DNA content [13], which is equivalent to an additional 3.2 chromosomes. This provides evidence for extensive genetic instability in the malignant MM B cells that overexpress HAS1 and its variants. Regardless of mechanism, the significant correlation between poor survival and the expression of HAS1 and its splice variants by circulating B cells suggests a key role for expression of HASs by “stem cell” components of the MM clone that circulate in the blood and mediate malignant spread to distant
bone marrow sites.

In addition to the impact of HA in cancer cell migration/spread, and in mitosis, HA, and by extension HASs, may also play a role in the cancer cell response to therapeutic drugs. Toole and colleagues has shown that HA oligomers compromise drug resistance mechanisms of malignant cells [14]. The converse of this idea postulates that overexpression of HASs, and a presumptive consequent increase in HA synthesis, may lead to enhanced drug resistance. Our previous analysis of drug resistance in MM has shown that overall, bone marrow-localized plasma cells are largely drug sensitive, while earlier stage MM B cells are largely drug resistant and persist in the circulation during and after chemotherapy (Figure 2) [15;16].

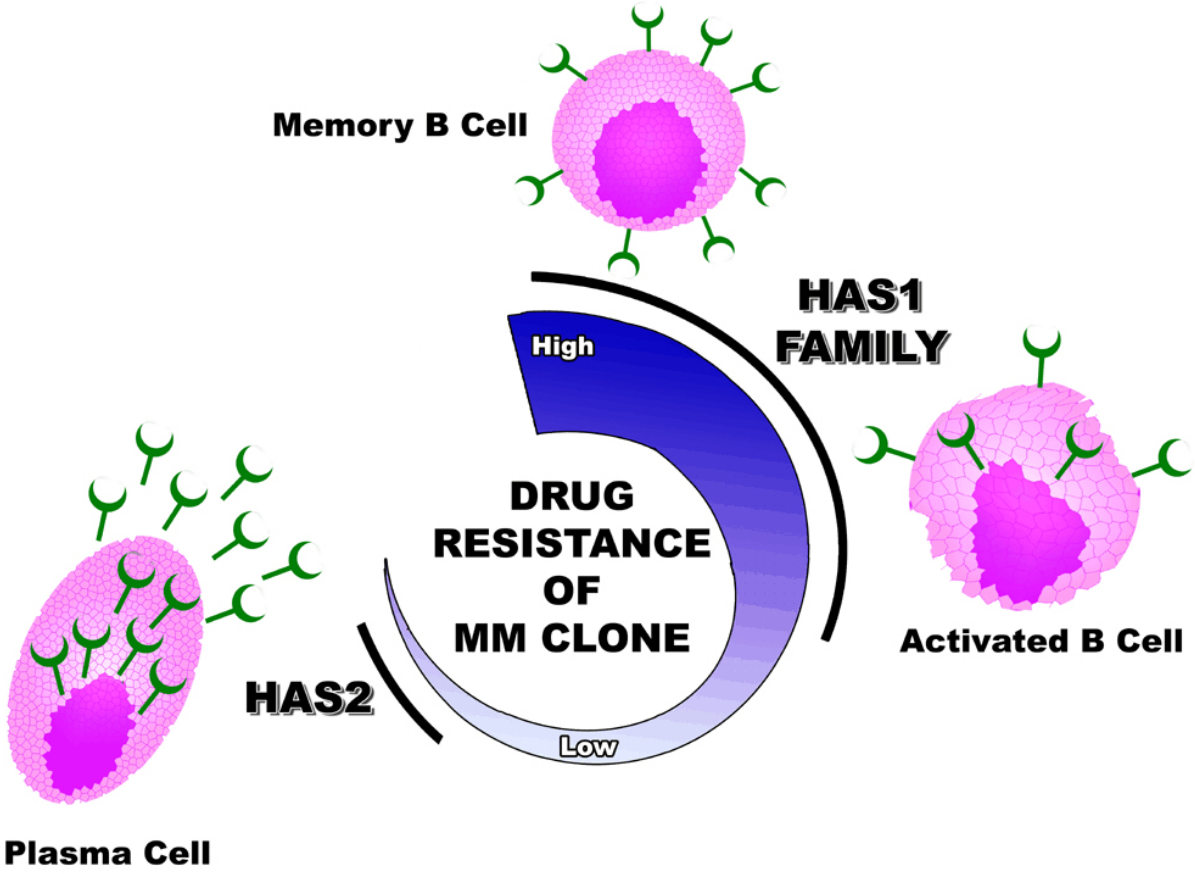

Figure 2: Therapeutic drug resistance correlates with expression of members of the HAS1 gene family, while drug sensitivity correlates with HAS2 gene expression. MM is comprised of B lineage cells at sequential differentiation stages indicated in the figure. Black lines indicate HAS gene expression. The blue ellipse indicates relative degree of drug resistance as determined by persistence of the indicated cell type throughout chemotherapy.

The detection of novel HAS1 variants in MM B cells at high levels and their absence from normal B cells, as well as from other cell types, suggests that aberrant HAS1 splicing is characteristic of malignant cells. The detection of HAS1Va in pre-malignant conditions (unpublished) suggests that its overexpression may be an early event in the genesis of myeloma. The enzymatically active part of full length HAS1 protein is largely intracellular. Based on

their sequences and predicted tertiary structures, we speculate that HAS1 variants are intracellular isoenzymes that retain enzymatically active domains that are likely to synthesize intracellular HA (Figure 3, Adamia et al., unpublished), a ligand for intracellular RHAMM, and thus contribute to the RHAMM-induced dysregulation of mitosis and subsequent chromosomal abnormalities.

The evidence above, coupled with our observations that treatment with HA triggers redistribution of intracellular RHAMM to the surface [17], leads to the speculation that HAS1 is a key regulator of RHAMM redistribution and that together, RHAMM and HAS1 control malignant spread. However, this may not be the only biologically important process in which RHAMM and HASs synergize to promote malignancy.

RHAMM and B lineage malignancy

RHAMM is a highly multifunctional protein first described and characterized by Turley and her coworkers [18-21]. Its carboxy terminal domains have been shown to bind HA [22] and erk kinase [23], and to target centrosomes [24]. The amino terminal domains, particularly the domains encoded by exon 4, bind microtubules [24-26]. RHAMM participates in embryogenesis, wound healing, inflammatory disease and oncogenesis. In human B lineage malignancies, RHAMM is found on the surface and in the cytoplasm of malignant B and plasma cells [27-30]. RHAMM protein is largely absent from non-malignant B and T lymphocytes from healthy donors, from T cells in MM patients [27;30;31], and from non-malignant BM plasma cells [32]. RHAMM cDNA [18] has a sequence distinct from CD44, an alternate HA receptor. The genomic sequence of murine RHAMM has 18 exons [19;33].

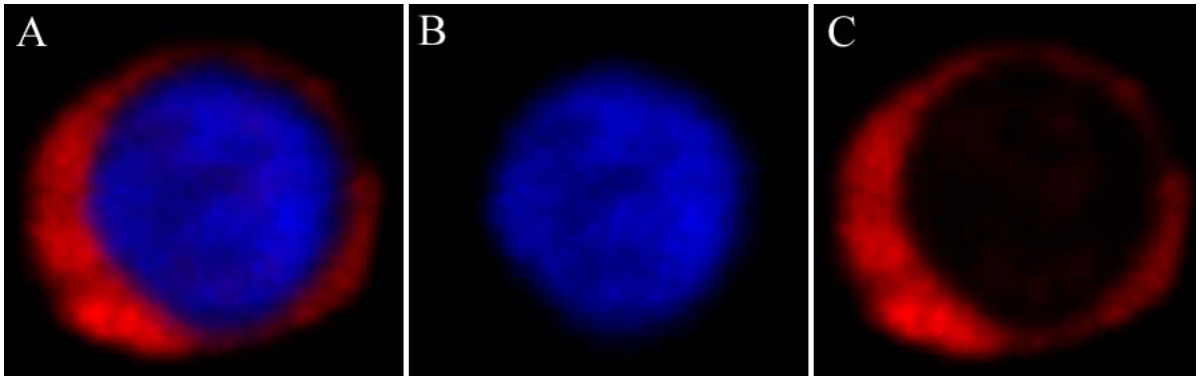

Figure 3: Intracellular HA in circulating malignant B cells from multiple myeloma patients.
Purified B cells from MM patients were stained with DAPI (blue, identifies nuclei) and with biotinylated HA binding protein (red, identifies HA), followed by streptavidin-Alexa594. Panel A is a composite image showing that HA (red) is detectable in the cytoplasm but not in the nucleus (blue). Panel B shows DAPI staining alone and Panel C shows staining with only biotinylated HA binding protein

Human RHAMM cDNA is overall 85% homologous to mouse RHAMM, with almost complete conservation of sequence in the HA binding domains [34]. RHAMM transcripts undergo alternative splicing to create at least 2 splice variants that are absent from healthy B cells but overexpressed in malignant B cells [27]. The splice variants isolated from malignant B and plasma cells lack exon 4 (a 48 bp deletion) or lack exon 13 (a 147 bp deletion). In lymphoid

cancers, full length RHAMM and the exon 4 deletion variant ($RHAMM^{-exon4}$) are frequent isoforms, with relatively low but consistent expression of the exon 13 deletion variant ($RHAMM^{-exon13}$) [27]. In individual plasma cells from MM patients, $RHAMM^{FL}$ and $RHAMM^{-exon4}$ isoforms are rarely coexpressed, suggesting that dysregulation of RHAMM splicing leads to emergence of malignant clones restricted to $RHAMM^{-exon4}$ expression, and increasing levels of $RHAMM^{-exon4}$ in the circulation herald pending relapse [32]. Overexpression of the $RHAMM^{-exon4}$ splice variant in MM patients correlates strongly with reduced survival [32] suggestive of an important role for RHAMM in malignant progression. Normal B lineage cells lack detectable surface RHAMM [30] and intracellular RHAMM [36], and for the most part lack detectable RHAMM transcripts (unpublished, [27;28]. In healthy people, RHAMM is detectable on a subset of thymocytes [17;31;37;38], and a subset of quiescent BM progenitor cells [39]. In healthy adults, the lymphocytes and stem cells expressing RHAMM appear to be non-dividing (unpublished).

RHAMM is an acidic coiled coil protein. On malignant cells, surface RHAMM binds HA [28;30;40], while intracellular forms interact with microtubules [25], centrosomes and the mitotic spindle [24]. HA binding by basal levels of surface RHAMM stimulates redistribution of intracellular RHAMM to the cell surface [17], indicative of an intimate link between surface-localized and intracellular pools of RHAMM. We speculate that RHAMM overexpression may enable avoidance of anoikis (cell death arising from loss of integrin mediated cell contacts, termed homelessness [41], thus facilitating survival of malignant cells. Overexpression of RHAMM isoforms on the surface of cancer cells may potentiate survival of the malignant cells, while intracellular forms of RHAMM contribute to extensive chromosomal instability by transiently compromising separation of the mitotic spindles, consistent with the pervasive and complex chromosomal abnormalities found in many cancers, particularly MM (e.g., [13;42-45]. Thus, overexpressed RHAMM appears to have a multifaceted impact on malignant progression through its functional roles in motility, that lead to malignant spread, and in mitosis lead to the generation of genetic abnormalities that have the potential to facilitate malignant progression. Therapeutic ablation of RHAMM is predicted to stabilize mitosis and promote cell death, as well as halting malignant spread, thus perhaps arresting malignant progression. Furthermore, although the role of angiogenesis in blood cancers is still controversial, RHAMM appears to play a role in angiogenesis, as anti-RHAMM antibodies block angiogenesis [46], potentially providing yet another means through which RHAMM potentiates malignancy.

The localization of RHAMM to both surface and cell interior [28;30;31;37;40], and the HA-regulated redistribution between sites [17;17;28], has led to the term “itinerant” receptor for RHAMM [47]. Deletion constructs that lack the HA binding domains are unable to associate with the centrosome [24]. Thus, the domains of RHAMM that bind HA and function on the cell surface may also be responsible for the centrosomal localization of RHAMM. Clearly, this region of RHAMM mediates multiple functional roles.

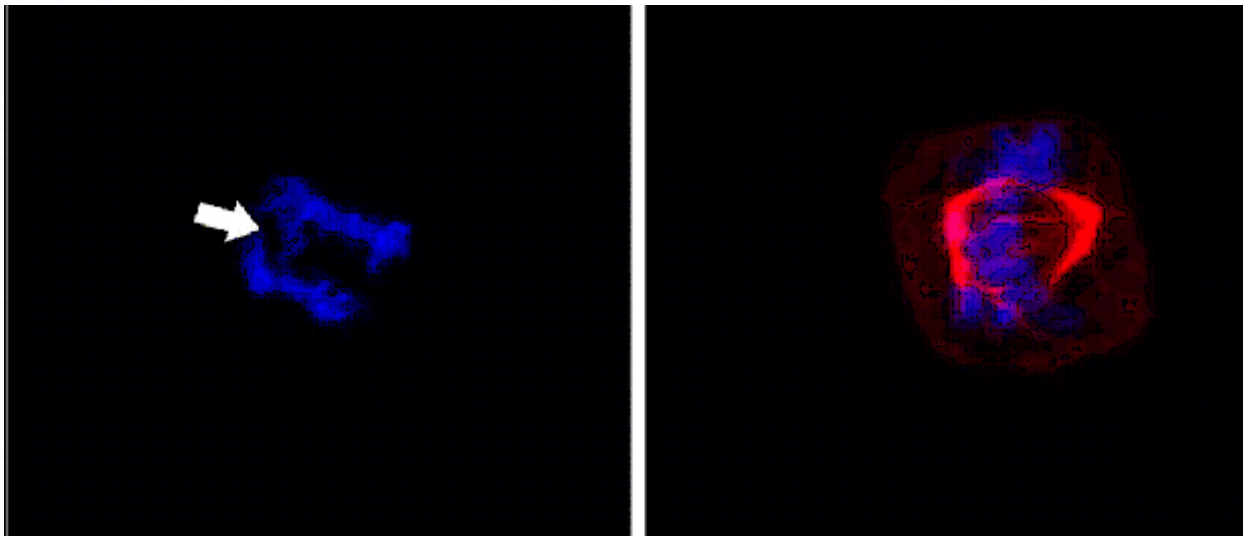

Figure 4: Chromosomal missegregation in RHAMM-transfected cells
RPMI8226 cells were transiently transfected with RHAMM constructs and then overexpress RHAMM by about 5 fold as compared to cells transfected with a vector control. Two cells in abnormal mitosis are shown. Chromosomes are stained with DAPI (blue). In the panel on the right, the red dye identifies transfected RHAMM.

Analysis of RHAMM-GFP transfectants suggests that all three RHAMM isoforms have surface localized compartments (Gares et al., in preparation). RHAMM^{-exon4} appears to be upregulated as Analysis of RHAMM-GFP transfectants suggests that all three RHAMM isoforms have surface localized compartments (Gares et al., in preparation). RHAMM^{-exon4} appears to be upregulated as MM worsens, and relative overexpression of RHAMM^{-exon4} as compared to RHAMM^{FL} is significantly correlated with poor survival in MM [32]. Based on analysis of RHAMM-GFP constructs, RHAMM^{-exon4} lacks avid binding to microtubules, but retains the ability to associate with the centrosome and spindle [24]. Overexpression of RHAMM in transfectants, at levels that are comparable to those detected in freshly isolated MM cells (in the range of 3-6 fold overexpression) results in centrosomal abnormalities similar to those in MM, and chromosomal missegregation (Figure 4). Microinjection of anti-RHAMM to deplete endogenous RHAMM also leads to abnormal mitoses [24]. Thus, both over- and under-expression of RHAMM disrupts mitotic events with potential generation of chromosomal missegregation and aneuploidy. This supports the hypothesis that RHAMM abnormalities in MM may underlie the extensive chromosomal instability that characterizes this disease.

Interactions between RHAMM and HAS1: A potential mechanism of chromosomal instability in human cancer

RHAMM appears to play a role in B lymphoid malignancies [27;30;40], as well as breast [48;49], stomach [50] and colorectal [51] cancers, making analysis of its functional role a high priority. Our working hypothesis predicts that in B cell malignancies, endogenous synthesis of HA exerts control over RHAMM localization patterns inside the cell and on its surface. Thus, therapeutic alterations in intracellular HA, or in the ability of HA binding domains of RHAMM to interact with the mitotic apparatus, may provide a means to abrogate the effects of overexpressed RHAMM. Like RHAMM, HA itself is itinerant, on the cell surface and inside the cell. Proliferating cells have intracellular HA, some of which is internalized HA and some of which is synthesized intracellularly, each with distinct physical distribution patterns [52].

Furthermore, in the systems so far analyzed, both surface and intracellular forms of RHAMM efficiently bind HA [39]. Finally, the carboxy terminal domain of RHAMM include both HA binding domains and the centrosomal targeting region [24], suggestive of potential overlap and the possibility for competitive inhibition by intracellular HA of centrosomal targeting by RHAMM. This means that inside the cell, cytoplasmic HA may interfere/compete with centrosomal associations of intracellular RHAMM, and if present in excess on the cell surface may lead to deregulation of proliferation and cell behavior.

In addition to promoting RHAMM-dependent motility of malignant cells, HAS1 and its novel variants may also synergize with RHAMM to promote the emergence of increasingly aggressive genetic variants in MM. Abnormalities in RHAMM isoforms, RHAMM expression levels, and overexpression of HAS1 and/or HAS1 variants may determine the ultimate balance between an outcome of apoptosis and death, or of chromosomal instability, viability and clonal emergence. *In vivo*, too much or too little RHAMM during mitosis, coupled with aberrant HAS1 function, may lead to more aggressive disease.

There exist provocative mechanistic links between RHAMM and HASs:

- MM B cells have clinically significant expression of HAS1 and HAS1 splice variants.
- MM B cells express intracellular HA.
- The C terminal domains of RHAMM that mediate centrosomal targeting overlap the HA binding domains.
- Intracellular HA may compete for centrosomal targeting sites of RHAMM.

It is thus reasonable to speculate that the malignant cells comprising the myeloma clone acquire an increasing degree of chromosomal instability as a result of abnormal RHAMM and HAS expression as follows:

- RHAMM-mediated centrosomal abnormalities lead to chromosomal instability.
- High levels of RHAMM may abort mitotic events, leading to cell death in the absence of other abnormalities.
- Aberrant HAS1 splicing leads to production of intracellular HA, competition for centrosomal targeting sites and rescue of malignant daughter cells that would otherwise undergo apoptosis caused by mitotic arrest in the presence of high levels of RHAMM.
- HAS1 variant-enabled rescue of RHAMM overexpressors thus facilitates survival and emergence of aggressive variant clones, leading to progressive disease and short survival.

RHAMM and HASs thus represent a new type of prognostic marker that reflects biologically important properties of the malignant clone as it undergoes stepwise oncogenesis and/or disease progression.

At the intracellular level, the impact of RHAMM and HASs could be manifested as increased genetic abnormalities with direct oncogenic potential and/or by predisposing cells to additional oncogenic changes that may enable proliferation despite abnormal chromosomal complements. Modulation of overexpressed RHAMM and increasingly dominant expression of the $RHAMM^{-}_{exon4}$ isoform, in the context of intracellular HA made by abnormally expressed and abnormally spliced HAS1, may be a strong contributor to the extensive, complex and progressively

increasing degree of chromosomal abnormalities seen in MM. Our experimental observations suggest that in myeloma patients, the overexpression of RHAMM isoforms together with overexpression and aberrant splicing of HAS1 may potentiate the malignant process by contributing to extensive chromosomal instability, avoidance of cell death and disease dissemination.

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