RASSF1A and the rs2073498 cancer associated SNP

Howard Donninger1, Thibaut Barnoud1, Nick Nelson1, Suzanna Kassler1, Jennifer Clark1, Timothy D. Cummins2, David W. Powell2, Sarah Nyante3, Robert C. Millikan3 and Geoffrey J. Clark1*

1 Molecular Targets Program, Department of Medicine, James Graham Brown Cancer Center, University of Louisville, Louisville, KY, USA
2 Department of Biochemistry and Molecular Biology, University of Louisville, Louisville, KY, USA
3 The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

INTRODUCTION

RASSF1A is a tumor suppressor that suffers epigenetic inactivation in over 50% of human tumors (Dammann et al., 2005; Donninger et al., 2007; van der and Adams, 2007). Knockout RASSF1A mice demonstrate an enhanced predisposition to develop spontaneous tumors (Dammann et al., 2000) and this effect is amplified in a p53 null mouse background (Tommasi et al., 2011). RASSF1A is pro-apoptotic and can induce cell cycle arrest in G2 and G1 (Shivakumar et al., 2002; Vos et al., 2004, 2006). RASSF1A has no enzymatic activity, but appears to act by modulating microtubule polymerization and by scaffolding other tumor suppressors. Several tumor suppressor pathways are now known to be directly modulated by RASSF1A including MOAP-1/Bax (Baksh et al., 2005; Vos et al., 2006), the hippo pathway (Guo et al., 2007), and p53 (Song et al., 2008).

KEY RASSF1A INTERACTIONS

RASSF1A and K-Ras

RASSF1A contains a Ras association domain and a cysteine rich domain (CRD). By analogy to the classic Ras effector Raf, both of these domains have the potential to bind directly to Ras oncoproteins (Drugan et al., 1996). RASSF1A can be detected in an endogenous complex with Ras (Calvisi et al., 2006), can form a complex with exogenously expressed activated K-Ras and serve as pro-apoptotic K-Ras effector (Vos et al., 2006; Donninger et al., 2007). There remains some controversy over the physiological nature of the interaction between Ras and RASSF1A. This may have arisen because only the K-Ras specific isoform interacts with RASSF1A, and the K-Ras protein must be post-translationally modified to support the interaction (Donninger et al., 2007). Moreover, our experience working with the Ras effector Raf-1 showed us that inclusion of EDTA or strong detergents in the binding buffer can destroy the interaction between Ras and CRD structures (data not shown).

RASSF1A and microtubules

Early work showed that RASSF1A forms a complex with the microtubule network in cells and co-immunoprecipitates with most tubulin isoforms. Moreover, the interaction with RASSF1A appears to promote a robust, Taxol-like increase in the polymerization of microtubules (Liu et al., 2003; Dallol et al., 2004; Vos et al., 2004). Yeast two-hybrid studies have found that RASSF1A can directly bind a series of microtubule associating proteins (MAPs) including MAP1a/MAP1b, C19ORF5 (Dallol et al., 2004), and MAP4 (Vos et al., 2004). Thus, it seems likely that the association with microtubules is indirect. Interestingly, certain tumor derived mutants of RASSF1A appear to lose the ability to associate with specific tubulin isoforms (El-Kalla et al., 2010). Moreover, deletion mutants of RASSF1A that lose the ability to complex with microtubules are defective for the induction of cell cycle arrest (Vos et al., 2004). These data suggest that the interaction of RASSF1A with microtubules may be unusually complex and essential for its tumor suppressing activity.

RASSF1A and MOAP-1/Bax

MOAP-1 is a BH3 (Bcl-2 homology3) domain containing protein that binds and activates the pro-apoptotic effector Bax (Tan et al., 2005). RASSF1A was found to bind directly to MOAP-1 and thereby stimulate Bax apoptotic activity (Baksh et al., 2005; Vos et al., 2006). As MOAP-1 can associate with TNF-R1 and TRAIL-R1/TR4 death receptors, this links RASSF1A into TNF alpha/TRAIL death signaling pathways (Baksh et al., 2005). The interaction of RASSF1A and MOAP-1 is enhanced by the presence of activated K-Ras. Thus RASSF1A can act as a connector between...
RAS and Bax. Cells knocked out for RASSF1A exhibit reduced Bax activation and apoptosis when transfected with activated K-Ras (Baksh et al., 2005; Vos et al., 2006).

**RASSF1A and the Hippo pathway**

RASSF1A directly binds and activates the pro-apoptotic MST1 and MST2 kinases (Praskova et al., 2004; Avruch et al., 2006). These kinases drive the hippo pathway by phosphorylating and kinases LAT1 and LAT2 (Harvey and Tapon, 2007). These kinases then phosphorylate the transcription factors YAP and TAZ, which in turn activate the p73 tumor suppressor (Matallanas et al., 2007). The full role of RASSF1A in this pathway may be more complex as it also directly binds a protein called Salvador. Salvador may serve as an adaptor to promote the phosphorylation of LATs by MST (Guo et al., 2007). However, Salvador also appears to be a tumor suppressor in its own right, with significant functions independent of the classic hippo pathway (Donninger et al., 2011).

In summary, we now know that RASSF1A can complex with multiple known tumor suppressors and modulate their activity. This gives RASSF1A the potential to serve as a tumor suppressor node, integrating the activity of multiple tumor suppressors and connecting K-Ras to their action.

**THE rs2073498A SNP**

Several SNPs have been identified in the RASSF1A gene. The best characterized (rs2073498) is a C–A variation resulting in the presence of a Serine instead of an Alanine at amino acid 133 in the RASSF1A protein. This A133S variant protein has been associated with a reduced ability to regulate the cell cycle at G1/S (Shivakumar et al., 2002). Structurally, the A133S variant falls in a rather an intriguing position in the RASSF1A protein, within the minimal microtubule association domain (residues 120–185; Vos et al., 2004). Although the A(133)S variant retains an overly similar association with microtubules as the wild type protein, it is defective for the interaction with gamma and alpha (but not beta) tubulin (El-Kalla et al., 2010). Moreover, the change of Alanine133 to a Serine destroys a consensus phosphorylation site for the ATM kinase, an essential component of the DNA damage response (Hamilton et al., 2009). In fact it generates a new consensus phosphorylation site for several other kinases, including Casein kinase II. Thus, the biology and the regulation of the A(133)S variant may be significantly different to that of the wild type protein.

**THE rs2073498 SNP AND CANCER PREDISPOSITION**

In 2005, an analysis was performed to determine if SNPs detected in RASSF1A might have any association with the development of cancer (Schagdarsurengin et al., 2005). A striking result was obtained showing that the rs2073498 SNP was present in approximately 2% of non-cancer patient controls but almost 20% of breast cancer patients. A subsequent study (Gao et al., 2008) showed a much more modest increased association of the SNP with breast cancer development but linked it to a predisposition to early onset of disease in BRCA1 mutant positive patients. The presence of the SNP in the normal control population was found to be dramatically higher in this study (~18%). Moreover, the dbSNP (Sherry et al., 2001) gives a frequency of ~29% for the SNP in normal European populations. A third study (Bergqvist et al., 2010) has now been performed and has been unable to confirm a link between the SNP and breast cancer predisposition. However, this study did not address age of onset issues. This study also found a much higher frequency of the SNP in their non-cancer controls. Thus, there remains a controversy regarding the importance of the SNP for the development of breast cancer.

Here we have sought to address the role of the rs2073498 SNP in RASSF1A function and in breast cancer predisposition. We have compared the biological characteristics of the wild type and SNP derived proteins and identified differences in protein/protein interactions as well as in the effects of the proteins on cellular adhesion. We have also screened a larger cohort of breast cancer patients for the presence of the SNP. However, we found only a very modest increase in breast cancer amongst carriers of the SNP.

### RESULTS

**FREQUENCY OF THE rs2073498 SNP IN BREAST CANCER**

We have analyzed the largest breast cancer population group to date for the presence of the rs2073498 C/A SNP in RASSF1A. This group consisted of a primarily Caucasian, Non-African American (NAA) group of 1118 normal, and 1230 breast cancer patients as well as an African American (AA) group of 658 normal and 742 breast cancer patients (Table 1). We find that 20% of the normal NAA population are Heterozygous, and 2% are Homozygous, for the allele. Curiously, only ~6.1% of 658 normal AA women carried the allele. This shows that there is a dramatic racial disparity in the presence of the allele. We observed a very modest 4.5% (odds ratio 1.066) increase in frequency for the presence of the SNP in NAA populations with breast cancer. This is not considered statistically significant (P-value 0.6). No increase was observed in the much smaller AA sample. In fact, the SNP was less present in the AA breast cancer patients.

| RASSF1A | Codon133Ala/Ser | rs 2073498 |
|---------|----------------|-----------|
| Ser (S) = A | A/A 20 (2%) A/A 20 (2%) | A/A 0 (0%) A/A 20 (2%) |
| Ala (A) = C | C/C 713 (96%) C/C 616 (94%) | C/C 713 (96%) C/C 616 (94%) |
| Allele frequencies | C = 0.98 C = 0.97 | A = 0.02 C = 0.97 |
| Genotype frequencies | C/A 29 (4%) C/A 41 (6%) | A/O A/A 10 (1.0%) |
| Hardy-Weinberg test P-value | 0.59 0.71 | 0.59 0.71 |
| Non-African Americans | Cases (N = 1230) Controls (N = 1118) | Cases (N = 1230) Controls (N = 1118) |
| Allele frequencies | C = 0.88 C = 0.88 | A = 0.12 A = 0.12 |
| Chi-square P-value | = 0.03 0.004 | = 0.03 0.004 |
| Genotype frequencies | C/C 952 (77%) C/C 875 (78%) | C/C 952 (77%) C/C 875 (78%) |
| Chi-square P-value | C/A 258 (21%) C/A 223 (20%) | A/A 20 (2%) A/A 20 (2%) |
| Hardy-Weinberg test P-value | 0.60 0.19 | 0.60 0.19 |
When breaking up the NAA breast cancers by sub-type, the SNP was present in 22% of normal control samples and 26.3% of Luminal A breast cancer patients \( (P\text{-value} = 0.094, \text{odds ratio} \ 1.4) \), no differences were detected in Luminal B or Triple negative patients. Moreover, no significant \( (P = 0.85) \) differences in age of onset were observed (Table 2). However, data was not available to consider the role of the BRCA1 mutation status in the samples.

We have also performed a series of experiments in an attempt to determine if there are significant differences in the biological activity of between the wild type RASSF1A protein and the A(133)S variant produced by the SNP allele that might support a role in cancer predisposition.

### THE RASSF1A A(133)S VARIANT ALTERS CELLULAR ADHESION

In order to examine any potential differences in biological activity between the wild type RASSF1A and the A(133)S variant, we transfected H1299 lung carcinoma cells (RASSF1A negative) with expression constructs to generate stable cell lines expressing approximately equal amounts of each protein (Figure 1). We found little obvious difference in the rate of cell growth between the matched pair of cells. However, we detected a significant increase in the degree of adhesion in the A(133)S variant compared to the wild type RASSF1A expressing cells (Figure 1). Whereas transformed cells often exhibit reduced adhesion which correlates with enhanced motility, here we found that the variant RASSF1A expressing cells demonstrate stronger adhesion to the substratum than the wild type RASSF1A expressing cells. However, there are multiple examples where enhanced adhesion has been associated with enhanced transformation (Kenny et al., 2008) or survival (Tsujii and DuBois, 1995).

| Codon 133 status | Number of patients | Mean age of onset |
|------------------|--------------------|-------------------|
| C/C              | 952                | 51.323            |
| C/A + A/A        | 278                | 51.003            |

T-tests give a \( P\text{-value} = 0.85 \).

**Table 2 | Average age of onset of breast cancer in wt vs. rs2073498 SNP patients.**

**FIGURE 1 |** The A(133)S SNP induces enhanced adhesion. (A) H1299 cells stably transfected with HA tagged RASSF1A expression vector were assayed for the strength of their adhesion to culture dishes. The difference between the wild type and the A(133)S variant transfected cells was statistically significant \( (P = 0.03) \). (B) Levels of RASSF1A expression in the stable cell lines was confirmed by western analysis using an HA antibody.

- **TABLE 3 |** Summary of proteins identified as demonstrating differential interactions with the wild type and A(133)S variant of RASSF1A.

| Protein target | RASSF1A WT | RASSF1A A133S |
|----------------|------------|--------------|
| Tau            | 0.26       | 0            |
| AGR3/ECMP11    | 0          | 0.5          |
| ANAPC7         | 0          | 0.15         |
| Claudin 10     | 4          | 0            |
| Vimentin       | 0.55       | 0            |
| RASSF3         | 0.36       | 0            |
| Map1a          | 0.68       | 0            |
| Ksr            | 0.07       | 0.0          |
| MST1           | 0.1        | 0.1          |
| C19orf5        | 0.7        | 0.7          |
| RASSF1A        | 4.3        | 3.8          |

Quantification of interactions is shown as protein abundance factor (PAF). Essentially this value is calculated from the total of unique MS/MS spectra that identified each individual protein divided by the molecular weight (Dalton) \( \times 10^4 \). Proteins denoted in blue are proteins that are already known to bind directly to RASSF1A. Therefore, their presence in the screen at equivalent levels serves as an internal control. The levels of RASSF1A expressed during the transfection can also be seen to be similar (denoted in red).

**FIGURE 2 |**

**DISCUSSION**

RASSF1A is an important tumor suppressor with the potential to impact many different biological processes that are critical to the development of cancer. The A(133)S SNP derived protein has now been shown to exhibit a number of subtle defects in biological function. These include differential binding to various interaction partners, differential effects on the cell cycle and cellular adhesion. We have also recently observed a differential interaction with components of the DNA repair machinery and found that the A(133)S variant form of RASSF1A can act as

- **THE RASSF1A A(133)S VARIANT EXHIBITS DIFFERENTIAL BINDING TO A VARIETY OF POTENTIAL RASSF1A TARGETS**

In an attempt to identify proteins which bind differentially between the wild type and the SNP derived protein, we performed a proteomic analysis of immunoprecipitations of wild type and A(133)S RASSF1A transfected into 293-T cells. Problems in interpreting this type of experiment can arise because of the degree of background. However, here we were focusing on differences between the two forms of the protein rather than the total composition of the pull down. We identified a number of known RASSF1A interacting proteins that bound to the same degree, such as MST1 and C19orf5 (Table 3, denoted in blue), but we also identified a small group of proteins that appeared to exhibit differential association (Table 3). Some proteins appeared to gain association with the SNP derived variant, and some appeared to lose association.

We have begun to validate these results by Western blot. Our initial progress suggests that these results may be accurate as MAP1a appeared to show differential binding when we performed co-transfection/co-immunoprecipitation experiments (Figure 2). This is a specific defect, as MAP4 showed equivalent binding. Further validation is proceeding.
Vimentin overexpression in cancer has been documented and a potential role in metastasis identified (Satelli and Li, 2011). Vimentin has been implicated in adhesion (Ivaska et al., 2007) and so the differential association might explain the differential adhesion promoted by the two forms of RASSF1A (Figure 1).

The A(133)S variant of RASSF1A has previously been shown to be defective for the interaction with gamma and alpha tubulin, although the interaction with beta tubulin was retained (El-Kalla et al., 2010). This implies that it is defective for the interaction with MAPs which are already known to bind directly to RASSF1A (Dalbol et al., 2004). Potential differences in the interaction with the MAPs Tau and MAP1a were identified in the screen. The defective interaction with MAP1a was confirmed by co-immunoprecipitation studies.

Ksr is thought to act as a scaffold facilitating the activation of the Raf oncoprotein by Ras. Ksr can complex with a second Raf scaffold called CNK (Claperon and Therrien, 2007). CNK has been reported to be able to bind RASSF1A and to mediate some of the apoptotic effects of RASSF1A (Rabizadeh et al., 2004).

RASSF3 remains the most obscure member of the RASSF family (Tommasi et al., 2002). In our hands it is pro-apoptotic but we have been unable to detect any loss of mRNA or protein expression in human tumor cells (data not shown). It has, however, been implicated in mediating resistance to transformation by deregulated Her/Neu in breast cancer (Jacquemart et al., 2009).

ANAPC7 is a sub-unit of the anaphase promoting complex (APC). The APC acts to regulate the levels of a variety of key components of the cell cycle. RASSF1A has previously been implicated in modulating APC activity via binding cdc20 (Song and Lim, 2004). However, this interaction remains controversial (Liu et al., 2007).

AGR3/BCMP11 remains a relatively obscure protein but has recently been associated with differentiation and survival in ovarian cancer (King et al., 2011). It has also been identified as an over-expressed protein in breast cancer (Adam et al., 2003). Further validation, including endogenous co-immunoprecipitations, will be required to validate the data set.

An important point that may be worth considering is that when tumors lose expression of RASSF1A by promoter methylation, a smaller isoform of RASSF1 called RASSF1C usually remains (Richter et al., 2009). In these cells, the SNP will manifest at a protein level as RASSF1A A(133)S that may be driving any cancer susceptibility phenotype. Indeed, some evidence has been presented that RASSF1C can serve as an oncogene (Reeves et al., 2010).

A further consideration is that the SNP shows a dramatic difference in its occurrence between different racial populations. According to the dbSNP (Sherry et al., 2001), the frequency of the SNP is almost 15 times higher in European populations than in African populations. Our studies based on populations in the USA found an approximately fourfold higher frequency in NAAAs than in AAs. Therefore, to determine if the SNP predisposes toward cancer, perhaps we should focus on cancers that are more frequent in the NAA populations such as ovarian (Ness et al., 2000). This observation also means that if study populations are not controlled for race, then any epidemiology results obtained may be seriously flawed.
MATERIALS AND METHODS
PLASMIDS AND DNA
RASSF1A plasmids have been described previously (Dallol et al., 2004; Vos et al., 2006). GFP–MAP1A was a kind gift of Chien et al., 2005; Chung Shan Medical University, Taichung, Taiwan).

TISSUE CULTURE AND WESTERN ANALYSIS
293-T cells were cultured and transfected as described previously Vos and Clark (2005). After 24 h, transfected cells were lysed as described previously Vos and Clark (2005) and immunoprecipitated with GFP-trap beads (Allele Biotech, Belgium) according to the manufacturer’s instructions. The immunoprecipitates were Western blotted with anti-GFP (Santa Cruz Bio, Santa Cruz, CA, USA) and anti-HA antibodies (Sigma, St. Louis, MO, USA).

ADHESION ASSAYS
H1299 cells (ATCC, Manassas, VA, USA) were stably transfected with RASSF1A wild type or mutant cloned in the vector pZIP-neoHA (Fiordalisi et al., 2001). Cells were selected in G418 and adhesion assays were performed as described in (Humphries, 2001). Essentially, cells were plated at 5 × 10^4 cells per well in 96-well plates and allowed to adhere for 45 min at 37°C. The medium was removed and the adhering cells fixed and stained with crystal violet. The dye was solubilized in 10% acetic acid and absorbance at 570 nm was used as a measure of adhesion.

PROTEOMICS
Ten 10 cm dishes of 293-T cells were transfected with 5 μg of HA tagged RASSF1A expression plasmid. Cells were lysed and immunoprecipitated with HA conjugated beads after 24 h. Samples were washed and subjected to trypsin digestion. Trypsin-digested samples were analyzed by 2D-LC-MS/MS. Briefly, protein samples were digested with modified trypsin (Promega, Madison, WI, USA) overnight at 37°C as previously described (Cummings et al., 2010). Resulting peptides were loaded onto an analytical 2D capillary chromatography column packed with strong cation exchange (SCX) and C18 reversed-phase (RP) resin (Phenomenex, Torrance, CA, USA). This biphasic column was attached to an analytical RP chromatography column with an integrated, laser pulled emitter tip. Peptides were eluted from SCX with seven step gradients of 5, 10, 15, 30, 50, 70, and 100% of 500 mM ammonium acetate. Following each SCX elution step, peptides were ionized and eluted into a linear ion trap mass spectrometer according to the following linear HPLC gradient: 20 min: 0% B, 80 min: 40% B, 90 min: 60% B at a flow rate of 200 nl/min (mobile phase A: 5% acetonitrile/0.1% formic acid and mobile phase B: 80% acetonitrile/0.1% formic acid). MS/MS spectra were acquired with a LTQ ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Protein identification was performed with Sequest Sorcerer (Sage-N Research, San Jose, CA, USA), which was set up to search a FASTA formatted human protein database (Human RefSeq) with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 1.2 Da. High-probability peptide and protein assignments from were made using Peptide and Protein Prophet algorithms (Neubizhiksii et al., 2003). Abundance of each identified protein was determined by normalizing the number of unique spectral counts matching to the protein by its predicted molecular weight. This value has been termed a protein abundance factor (Powell et al., 2004). Comparative analysis was performed with an in-house, web-based program (McAfee et al., 2006).

GENOMICS
Experiments were performed as described previously (Nyante et al., 2011). Essentially, the Carolina Breast Cancer Study (CBCS) is an IRB approved, comprehensive, interdisciplinary investigation into the causes of invasive and in-situ breast cancer among AA and white women in North Carolina. Enrollment was conducted in two phases, Phase 1 (1993–1996) and Phase 2 (1996–2001), and the total number of enrolled participants is 4333. Phase 1 and Phase 2 of the study includes 2311 cases (894 AA, 1417 NAA, primarily Caucasian) and 2022 controls (788 AA, 1234 NAA). Peripheral blood lymphocytes were collected and DNA extracted for 90% of participants. Genotyping was completed using the Illumina Golden Gate platform: 96% of samples had call rates greater than 95; 90% of SNPs were successfully genotyped; control DNA samples had concordance rates of 99.9%. SAS was used to calculate chi-squared tests and Hardy Weinberg tests.

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