A RASSF1A polymorphism restricts p53/p73 activation and associates with poor survival and accelerated age of onset of soft tissue sarcoma.

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Abbreviations
RASSF1A - Ras association domain containing family 1A, ATM- ataxia telangiectasia mutated, SNP - single nucleotide polymorphism.
Abstract

RASSF1A (Ras association domain containing family 1A), a tumor suppressor gene that is frequently inactivated in human cancers, is phosphorylated by Ataxia Telangiectasia Mutated (ATM) on Ser131 upon DNA damage, leading to activation of a p73-dependent apoptotic response. A single nucleotide polymorphism located in the region of the key ATM activation site of RASSF1A predicts the conversion of alanine (encoded by the major G-allele) to serine (encoded by the minor T-allele) at residue 133 of RASSF1A (p.Ala133Ser). Secondary protein structure prediction studies suggest that an alpha helix containing the ATM recognition site is disrupted in the serine isoform of RASSF1A (RASSF1A-p.133Ser). In this study, we observed a reduced ability of ATM to recruit and phosphorylate RASSF1A-p.133Ser upon DNA damage. RASSF1A-p.133Ser failed to activate the MST2/LATS pathway, which is required for YAP/p73 mediated apoptosis, and negatively affected the activation of p53, culminating in a defective cellular response to DNA damage. Consistent with a defective p53 response, we found that male soft tissue sarcoma patients carrying the minor T-allele encoding RASSF1A-p.133Ser exhibited poorer tumor-specific survival and earlier age of onset compared with patients homozygous for the major G-allele. Our findings propose a model that suggests a certain subset of the population have inherently weaker p73/p53 activation due to inefficient signaling through RASSF1A, which affects both cancer incidence and survival.
Introduction

*RASSF1A* (RAS association domain family 1 isoform A) is a tumor suppressor gene located on chromosome 3p21.3, an area that is frequently deleted in lung and breast cancers (1, 2). Although loss of heterozygosity (LOH) has been observed, RASSF1A is more frequently epigenetically inactivated by hypermethylation of CpG islands within the promoter and the first exon, limiting expression in a wide variety of human cancers (3-6). High promoter methylation levels (implying low RASSF1A mRNA and protein levels) have been shown to associate with poor prognosis and is thus considered an independent prognostic indicator of overall survival in lung, renal and breast cancers as well as in uveal-melanoma, hepatoblastoma and sarcoma (7-12). Interestingly, lower levels of *RASSF1A* transcript have also been correlated with the decreased radiochemosensitivity of hepatoblastoma and colorectal cell lines (7, 13).

We have previously shown that RASSF1A responds directly to radiation and chemotherapeutic drug induced DNA damage via the main sensor of double-strand breaks, ATM. Upon DNA damage, RASSF1A is phosphorylated by ATM on Ser131 leading to the sequential activation of MST2 and LATS1 Ser/Thr kinases, stabilization and activation of the YAP1/p73 transcriptional complex, and ultimately apoptosis (14, 15). Recently, a number of components in this pathway have also been shown to play roles in the regulation and activation of p53 upon cellular stresses such as DNA damage and oncogenic activation. RASSF1A was shown to partially contribute to p53-dependent checkpoint activation by blocking the ubiquitin dependent degradation of p53 by MDM2 (16). Meanwhile, MST1 was shown to promote apoptosis by regulating the deacetylation of p53 and thereby enhancing transcriptional activity...
In addition, both LATS1 and LATS2 have been shown to regulate mitotic progression and a p53-dependent G1 tetraploidy checkpoint (18, 19). Furthermore, in response to oncogenic stress, activation of LATS2 phosphorylates ASPP1 which shunts p53 to proapoptotic promoters and promotes the death of polyploid cells (20).

Sequence alterations in the RASSF1A gene have been identified in non-methylated tumors and cell lines; a number of which have been confirmed as mutations that inactivate protein function (6, 21). These fall into predicted functional domains proposed to exert RASSF1A biological effects, most notably the ATM consensus motif (22). A rare mutation of the ATM phosphorylation site, Ser131Phe, ablates the ability of RASSF1A to respond to DNA damage and inhibit cell growth (15). As with the majority of alterations observed in the RASSF1A sequence, Ser131Phe does not appear to be an inherited polymorphism that can be identified at significant frequencies in the population (22).

RASSF1 c.397G>T (rs2073498) is a high frequency single nucleotide polymorphism (SNP) with a minor allele frequency (MAF) of up to 17% in European populations (International HAPMAP 1,000 Genomes Project; Figure S1). It is located close to the recognition site for ATM phosphorylation and is non-synonymous, predicting the conversion of alanine to serine at residue 133 of RASSF1A (p.Ala133Ser). The minor(Ser) allele of RASSF1A p.Ala133Ser has been associated with an increased risk of breast cancer (23) and early onset breast cancer in BRCA1/2 mutation carriers (24) as well as increased lung cancer risk in Japanese (25) and Chinese populations (26).
Here we provide a molecular explanation for these association studies, whereby we demonstrate that the predicted protein product of the minor allele, RASSF1A-p.133Ser, is impaired in its ability to activate both p73 and p53 tumor suppressor signaling in cellular systems. In humans, we go on to demonstrate that the minor allele of RASSF1 c.397G>T associates with altered soft-tissue sarcoma incidence and survival, a tumor type that is clearly suppressed and regulated by the p53 pathway (27).
MATERIALS AND METHODS

Patient populations

Soft-tissue Sarcoma Patients. 121 patients (68 females and 53 males; ages 14-87 years; mean 56 years) diagnosed with soft-tissue sarcomas in the years 1991 to 2001 at the Surgical Clinic 1, University of Leipzig, Germany, and at the Institute of Pathology of the Martin-Luther-University Halle, Germany, were included in the study. The mean observation time was 41 months (range 2-198 months). 57 patients died from tumor-related causes within the observation time, 64 patients were still alive at the time of follow-up. All patients underwent surgical treatment, 79 patients received postoperative radio- and/or chemotherapy (fractionated radiation with a cumulative dose of 60.4Gy; combination treatment of Doxorubicin and Ifosfamide).

Statistical analysis

Survival analysis was performed using the Kaplan-Meier analysis and the Cox multivariate proportional hazards regression model (SPSS 16.0 software; SPSS Inc., Chicago, IL, USA). The Mann-Whitney test was used to compare the age at diagnosis in the cohorts (InStat 3 software; GraphPad Software Inc, La Jolla, CA, USA). Values for p<0.05 were considered significant.

Plasmids and reagents

HA-RASSF1A-p.133Ala, FLAG-RASSF1A-p.133Ala, FLAG-RASSF1A-p.131Phe and FLAG-MST2 have previously been described (15, 49). HA-RASSF1A-p.133Ser and FLAG-RASSF1A-p.133Ser were obtained by site-directed mutagenesis using the QuikChange II Site-Directed Mutagenesis
Kit (Stratagene) according to the manufacturer’s instructions. All chemical reagents were purchased from Sigma unless stated otherwise.

**Tissue Culture**

All cell culture reagents were purchased from Gibco, Life Technologies. U2OS and H1299 cells were purchased from Cancer Research UK, London or LGC Promochem (ATCC). U2OS Tet-On cells (purchased from Clontech, California) that inducibly expressed RASSF1A p.133Ala and RASSF1A p.133Ser upon doxycycline induction were established following puromycin selection as described in the manufacturers protocol. All cells were cultured in complete DMEM (DMEM, 10% fetal calf serum, 1 mM sodium pyruvate, 2 mM glutamine, 0.1mM MEM non essential amino acids, 100 U/ml Penicillin and 100 μg/ml streptomycin). Cells were cultured in humidified incubator with 5% CO₂ at 37°C. Where indicated, cells were treated with the tetracycline analogue, doxycycline (2μg/ml), cisplatin (20μM) or adriamycin (0.2μg/ml). Irradiations were carried out in a Caesium-137 irradiator (GmbH).

**Antibodies**

The following antibodies were purchased from the indicated companies; anti-Flag (M2 clone), anti-Flag M2 agarose, anti-ATM (A6218) and anti-α–tubulin antibodies from Sigma; anti-RASSF1A clone 3F3 (sc-58470), anti-p53 (DO1)(sc-126), anti-p21 (sc-6246), anti Krs-1/2 (sc-6211) and anti-α–Tubulin (sc-8035) from Santa Cruz Biotechnology; anti phospho-Mst1 (Thr183)/Mst2 (Thr180) (#3681), anti phospho-LATS1 (Ser909)(#9157) from Cell Signalling Technology; anti-HA (05-904) from Millipore; anti-MST2 (1943-1), anti-p73 (1636-1) and anti-GAPDH (2251-1) from Epitomics; anti-LATS1 (BL2212) (A300-478A) from Bethyl Laboratories,
Inc. and anti-PUMA (ab9643) from Abcam. Secondary antibodies coupled to horseradish peroxidase were purchased from Pierce Biotechnology.

**Immunoprecipitations and Western Blotting**

Cells were cultured in complete media containing 0.1% FCS for 16 hr before treatment or harvesting (as indicated). Immunoprecipitations and western blotting were performed as previously described (15).

**Cell Assays**

Clonogenic assays were performed as previously described (15).

Cell Viability Assay: Assays were carried out as previously described (15). Briefly, H1299 cells were transfected with pcDNA3 or with plasmids expressing RASSF1A-p133Ala or RASSF1A-p.133Ser together with pBabe Puro. 24 hr after transfection, cells were trypsinized, replated and exposed to puromycin to select for transfected cells before being treated with the indicated amount of cisplatin. 48 hr later, cell viability was determined using the resazurin assay. Viability experiments were performed in triplicate and repeated at least twice.

In-vitro kinase assay: ATM kinase assays were carried out as previously described (15). Briefly, Flag-ATM immunoprecipitates from GM16667 cells (50) were incubated with the substrates (FLAG-RASSF1A proteins immunopurified from H1299 tet-on inducible cells) together with radiolabeled ATP for 90 min at 30°C. The kinase reactions were then subjected to SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membrane and subsequent visualized using a BioRad Phosphorimagener. In-gel kinase assay: The MST2 in-gel kinase assay was carried out as previously described (49).
Results

RASSF1A p.133Ser fails to activate the MST/LATS/p73 pathway in response to DNA damage.

The RASSF1 c.397G>T SNP (rs2073498) describes a G to T nucleotide change in the coding sequence of RASSF1 which converts an alanine (GCG) to a serine (TCG) at position 133 of RASSF1A. Although the conversion of alanine to serine is a structurally conservative one, the stability of protein secondary structures such as $\alpha$-helices has been shown to decrease with increasing serine content (28). The 15 amino acids (residues 128-142) surrounding codon 133 of RASSF1A corresponds to residues 199-214 of Nore1A/RapL, a related RASSF protein. The crystal structure of Nore1A/RapL demonstrates that the secondary structure of residues 200-215 is $\alpha$-helical in conformation (29) and secondary structure prediction using the ‘scratch protein predictor’ analysis (30) suggests that the homologous residues in RASSF1A adopt a similar configuration (Figure S2A, Upper). Replacement of alanine at position 133 by serine is predicted to destabilise one $\alpha$-helical turn (Figure S2A, Lower). This suggests that there may be a local disruption of secondary structure in the vicinity of the ATM phosphorylation site (Ser131) in RASSF1A-p.133Ser, which might possibly affect its ability to be recognised as an ATM substrate.

To investigate the impact of a modification from Ala to Ser at codon 133, we expressed the RASSF1A-p.133Ala and RASSF1A-p.133Ser isoforms in U2OS osteosarcoma cells and evaluated their ability to associate with ATM. As observed previously (15), we find that DNA damage increases the association of ATM with RASSF1A-p.133Ala (Figure 1A). By contrast, RASSF1A-p.133Ser does not appear to be recruited to ATM upon DNA damage (Figure 1A) suggesting that the conversion of Ser133 to Ala affects the interaction of RASSF1A with ATM and thus might
affect phosphorylation of RASSF1A at Ser131. Indeed, we found that an anti phospho-Ser131 antibody was able to recognize RASSF1A-p.133Ala but not RASSF1A-p.133Ser following DNA damage which suggests a detrimental effect on Ser131 phosphorylation (Figure S2B). To verify that RASSF1A-p.133Ser displays defective phosphorylation, both isoforms were immunopurified from transfected cells and subjected to an *in vitro* ATM kinase assay to directly evaluate phosphorylation status. Figure 1B shows that DNA damage activated ATM fails to phosphorylate RASSF1A-p.133Ser as efficiently as RASSF1A-p.133Ala.

The defective phosphorylation of RASSF1A-p.133Ser suggests that ATM mediated signaling should also be affected in response to DNA damage. Therefore we compared the ability of RASSF1A-p.133Ala and RASSF1A-p.133Ser to activate the MST/LATS pathway after DNA damage, utilizing the stabilization of the transcriptional co-factor YAP1 as a read-out. As predicted, we found that both cisplatin and ionizing radiation lead to increased YAP1 levels in the presence of RASSF1A-p.133Ala but not RASSF1A-p.133Ser (Figure 1C). To determine a functional consequence on YAP1 function, we evaluated the induction of the pro-apoptotic gene, *PUMA*, which is a known target of YAP/p73 transactivation in the absence of p53 (14). In H1299 cells (p53-null) treated with cisplatin, expression of RASSF1A-p.133Ala enhances PUMA induction compared to the control whereas RASSF1A-p.133Ser has no effect, indicating that the Serine isoform is indeed impaired in its ability to propagate a DNA damage signal (Figure 1D).

Previous studies have demonstrated that overexpression of RASSF1A-p.133Ala can overcome the requirement for signal dependent activation of the MST/LATS tumour suppressor pathway (14, 31-33). Upon testing the abilities of both RASSF1A isoforms to activate Flag-MST2,
we find that autophosphorylation of MST2 on Thr180 is stimulated by co-expression with RASSF1A-p.133Ala but not RASSF1A-p.133Ser (Figure 1E) suggesting that RASSF1A-p.133Ser has an intrinsic defect in its ability to activate the MST/LATS pathway. In keeping with this, we observed that stabilisation of YAP1 upon coexpression of MST2 and LATS1 was enhanced by RASSF1A-p.133Ala but not by RASSF1A-p.133Ser (Figure S3). Similarly, the expression level of p73 was enhanced when RASSF1A-p.133Ala, but not RASSF1A-p.133Ser, was co-transfected (Figure S4).

To confirm these results, we also examined the effect of these two isoforms on endogenous proteins. First, we examined the kinase activity of endogenous MST2 utilizing MBP (myelin basic protein) as a substrate in the presence of [γ-32P] ATP in an in gel kinase assay. Figure 1F demonstrates that phosphorylation of the MBP substrate, and hence the kinase activity of MST2, is significantly enhanced in the presence of RASSF1A.p133Ala but not RASSF1A.p133Ser. Downstream of MST2 activation, we also evaluated the association between MST2 and LATS1 in co-immunoprecipitation experiments. Formation of endogenous complexes between MST2 and LATS1 was stimulated by RASSF1A.p133Ala but again to a much lesser extent by the p.133Ser isoform (Figure 1G). It has previously been shown that MST2 phosphorylates LATS1 on two critical residues, Ser909 and Thr1079, resulting in LATS1 kinase activation (34). Western blot analysis of the immunoprecipitates with an antibody that specifically recognizes LATS1 on phospho-Ser909 (to monitor kinase activation) demonstrated that LATS1 appeared to be activated by MST2 only in the immunoprecipitated complexes from cells expressing the p.133 Ala isoform (Figure 1G), consistent with the data in Figure 1F.
To further validate the differential pathway activity, we investigated the interaction between YAP and p73 in the presence of the different RASSF1A isoforms. In agreement with previous observations (14), RASSF1A.p133Ala significantly enhances the endogenous YAP/p73 interaction; and as expected RASSF1A.p133Ser failed to elicit the same responses (Figures 1H). Taken together, these results suggest that RASSF1A-p.133Ser has an intrinsic defect that restricts activation of the MST/LATS/p73 pathway in addition to a disabled DNA damage response.

**RASSF1A-p.133Ser is defective in sensitising cells to DNA damaging agents**

To evaluate the physiological significance of RASSF1A p.Ala133Ser, we addressed the ability of the RASSF1A isoforms to affect cell viability after exposure to DNA damaging agents. H1299 cells (RASSF1A-null) were transfected with plasmids expressing either RASSF1A-p.133Ala or RASSF1A-p.133Ser, treated with cisplatin and evaluated for cell viability. In agreement with previous data (15), expression of RASSF1A-p.133Ala in H1299 cells results in increased sensitivity to cisplatin (Figure 2A). As expected, given its inability to promote p73 activation, RASSF1A-p.133Ser does not affect the cisplatin sensitivity of the cells compared to control-transfected cells (Figure 2A). To extend these observations to overall tumourigenicity, colony-forming assays were carried out with H1299 cells expressing RASSF1A-p.133Ala, RASSF1A-p.133Ser or RASSF1A mutated in the ATM phosphorylation site, RASSF1A-p.131Phe. While cells expressing RASSF1A-p.133Ala have a reduced ability to form colonies following ionising radiation, the growth of cells expressing RASSF1A-p.133Ser or RASSF1A-p.131Phe was
RASSF1A-p.133Ser restricts p53 activity in response to DNA damage.

RASSF1A, MST and LATS have been independently implicated in the regulation and activation of p53 in response to DNA damage (16-19). Indeed, depletion of RASSF1A in HeLa cells attenuates the stabilization of p53 and expression of its target genes in response to cisplatin (Figure S5). As DNA damage activation of MST2 and LATS1 is dependent on ATM phosphorylation of RASSF1A, we speculated that RASSF1A p.Ala133Ser may also affect the activation of p53. We first examined the effect of RASSF1A-p.133Ser on p53 protein stability. In cells treated with cycloheximide to inhibit protein synthesis, expression of RASSF1A-p.133Ala prolongs the stability of p53 to a greater extent than RASSF1A-p.133Ser (Figure 3A). In response to DNA damage, p53 is stabilised and activated which leads to the transcription and expression of p53 target genes such as p21 and PUMA. Upon treatment with Adriamycin, a DNA damaging agent, expression of RASSF1A-p.133Ala enhances the accumulation of p53 and p21 (Figure 3B). By contrast, expression of RASSF1A-p.133Ser resulted in diminished p53 stabilization and p21 expression compared to controls (Fig 3B). Complementary to these results, we find that adriamycin treatment leads to induction of PUMA protein expression which is enhanced in the
presence of RASSF1A p.133Ala but not the p.133Ser isoform (Figure 3C). This is consistent with the results for p21 expression in Figure 3B, lending support to the idea that the isoforms have differential effects on p53 transactivation activity. Fluorescence-activated cell sorting analysis was also carried out to monitor the cell cycle profile of U2OS cells with doxycycline inducible expression of RASSF1A-p.133Ala or FLAG-RASSF1A p.133Ser. In line with a previous report, induction of RASSF1A-p.133Ala in U2OS cells leads to a G1 delay in cell cycle progression (Figure 3D) (16). However, this was not observed in cells expressing RASSF1A.p133Ser (Figure 3D). Our results thus far indicate that the exchange of an alanine residue for serine at codon 133 not only impairs the ability of RASSF1A to transmit a DNA damage signal via ATM but also compromises its intrinsic ability to activate the MST/LATS/p73 and p53 tumour suppressor pathways.

**RASSF1 c.397G>T and promoter methylation are associated with poor survival outcome in patients with soft tissue sarcomas.**

Inherited mutational inactivation of one copy of the p53 gene in humans results in a dramatic increase in cancer risk (Li-Fraumeni syndrome), in which 90% of individuals with a mutation will develop at least one cancer by the age of 60, with soft tissue sarcomas (STS) being one of the most common (27). Higher frequency, less penetrant, inherited SNPs that affect p53 signaling have also been clearly shown to affect STS incidence and survival (35, 36). Our data thus far suggest that individuals carrying the minor T-allele of RASSF1 c.397G>T will have an attenuated p53/p73 signalling. We therefore wanted to address the impact of RASSF1 c.397G>T
on survival of STS patients after DNA damaging therapies. We studied a cohort of 121 German patients (clinical and histopathological data outlined in Supplementary Table S1), 79 of whom were treated with radiation and/or chemotherapy (RCHT) after surgical resection. As mentioned earlier, RASSF1 c.397G>T describes a transition in the coding DNA sequence and the patient cohort was genotyped accordingly, with the G and T-alleles designated as the major and minor alleles, respectively, whereby the T-allele encodes the RASSF1A-p.133Ser isoform. In this cohort, 107 (88.2%) patients were (G/G) in genotype, 11 (9.1%) patients were heterozygous (G/T) and 3 (2.7%) patients homozygous (T/T) for the SNP (Table 1); these percentages are comparable to the frequencies identified in the European populations of the 1,000 Genomes Project (Figure S1). There was no statistically significant difference in tumor-specific survival across the three genotypes in all 121 patients (data not shown). However, when segregated by gender, male patients homozygous (T/T) or heterozygous (G/T) for the minor T-allele displayed significantly shorter mean survival times compared to the G/G genotype (11.0, 50.5 & 100.9 months respectively, Kaplan Meier, log rank test p=0.016, Table 1 and Figure 4A) indicating an increasing trend towards poorer survival with RASSF1A-p.133Ser. Cox multivariate regression analysis was also performed to determine the effect on survival adjusting for known independent prognostic factors of STS, namely tumor stage and resection type (R-status) (37). A relative risk (RR) for tumor-related death of 4.88 was observed for male patients carrying at least one T-allele (Cox Regression, p=0.034, Table 1 and Figure 4B) suggesting a significant association between RASSF1 c.397G>T and survival outcome. By contrast, there was no significant difference in tumor-specific survival in female patients (45.3 vs 69.6 months, Kaplan
Meier, p=0.778; Cox Regression, RR=0.82, p=0.722, Table 1 and Figures 4A and B), suggesting a possible gender-specific effect of this locus.

Interestingly, the allelic difference in overall survival significantly increased when the multivariate regression analysis was restricted to a sub-set of STS patients that had been treated with radio and/or chemotherapy (RCHT). Male STS patients carrying the minor T-allele displayed significantly shorter survival times compared to patients with the G/G genotype (24.7 vs 68.6 months, p=0.039) with a significantly increased RR of 12.75 (Table 1 and Figure 4B), potentially indicating a poorer response to therapy. The female STS patients, however, do not show any significant difference in overall survival rates after RCHT (51.9 vs 63.0 months, Kaplan Meier, p=0.710; Cox Regression, RR=1.48, p=0.627, Table 1 and Figure 4B).

Taken together, these observations support the hypothesis that individuals carrying the minor T-allele of \textit{RASSF1} c.397G>T have attenuated RASSF1A signalling and therefore poorer survival after sarcoma diagnosis and subsequent treatment. These observations are also concurrent with our previous study, wherein we demonstrated that sarcoma patients whose tumours had attenuated RASSF1 signalling through epigenetic inactivation by hypermethylation of the promoter also associated with poorer prognosis (12). Interestingly, the significant association of the T-allele of \textit{RASSF1} c.397G>T with poor survival was only found in the male patients. To further explore the apparent sex-specific differences in responses to \textit{RASSF1} c.397G>T in overall survival of the patients in this cohort and to expand these analyses to include the well-documented epigenetic modification of RASSF1A in cancer cells, the methylation status of its promoter was included in the analyses. The methylation status of the
RASSF1A promoter was determined by MSP in 87 sarcomas from this cohort (12). Seventeen of the tumours were shown to contain hypermethylation of the promoter. There was no statistically significant difference in tumour-specific survival in all 87 patients when those patients with RASSF1A promoter methylation were compared to those without. Similarly to the associations with the genotypes of RASSF1 c.397G>T, when segregated by sex, the 6 male patients whose sarcomas contained a hypermethylated promoter displayed significantly shorter mean survival times compared to those 33 males whose sarcomas did not (22.5 & 65.8 months respectively, Kaplan Meier, log rank test p=0.002, Table 1 and Figure 4C). Cox multivariate regression analysis was also performed to determine the effect on survival adjusting for known independent prognostic factors of STS, namely tumor stage and resection type (R-status) (37). A relative risk (RR) for tumor-related death of 9.69 was observed for male patients whose sarcomas contained a hypermethylated promoter (Cox Regression, p=0.002, Table 1 and Figure 4D). By contrast, there was no significant difference in tumor-specific survival in the 11 female patients whose sarcomas contained a hypermethylated promoter compared to the 37 females who did not (82.3 vs 47.1 months, Kaplan Meier, p=0.368; Cox Regression, RR=0.75, p=0.644, Table 1 and Figures 4C and 4D). These associations appear to be for the most part independent of the RASSF1A-p.133Ser isoform as similar trends are observed in those 69 patients homozygous for the G-allele that encodes RASSF1A-p.133Ala and for which the methylation status was successfully determined. Specifically, the 4 male patients whose sarcomas contained a hypermethylated promoter displayed shorter mean survival times compared to those 28 males whose sarcomas did not (27.2 & 61.9 months respectively, Kaplan Meier, log rank test p=0.07, Figure 4D).
**RASSF1 c.397G>T is associated with increased incidence of STS at an earlier age in males.**

The results reported above support a model whereby RASSF1 c.397G>T affects the p53/p73 cellular responses, leading to decreased survival rates. However, as p53 and p73 signalling is also crucial in tumour suppression, we decided to explore the effect of RASSF1 c.397G>T on the age-dependent incidence of STS in these patients. Studies showed that patients carrying at least one copy of the minor allele were diagnosed on average 8.8 years earlier than patients G/G in genotype (48.6 vs 57.4 years, p=0.0851, Table 2). Interestingly, similar to the survival analyses, this difference in age at diagnosis was greatly increased when the analysis was segregated by gender, with males carrying the minor T-allele being diagnosed on average 22.2 years earlier than those G/G in genotype (33.7 vs 55.9 years, p=0.0098, Table 2 and Figure 5). By contrast, as with the survival analyses, no significant differences in age at diagnosis were observed in female patients (59.8 vs 58.5 years, p=0.962, Table 2 and Figure 5).

**DISCUSSION**

Our data indicates that the ability of RASSF1A to be activated by ATM is a key step for the pathway to influence tumor suppressor activity. The protein product of the minor T-allele, RASSF1A-p.133Ser, is predicted to have an altered secondary structure surrounding the ATM site, compared to RASSF1A-p.133Ala. In line with the variation in structure, we observe that RASSF1A-p.133Ser fails to be engaged by ATM and is concomitantly not phosphorylated upon DNA damage (Figures 1A and 1B). This suggested that the downstream effects of RASSF1A in response to DNA damage might be impaired and we show that this is indeed the case.
Interestingly, a previous report had shown that inactivation of the ATM phosphorylation site in RASSF1A (Ser131Phe or Ala133Ser) led to lower steady state phosphorylation at Ser203 (38). It has been reported that RASSF1A function is dependent on phosphorylation of RASSF1A-Ser203 and that elimination of this phosphorylation site abrogates its ability to regulate cytokinesis, microtubule stability and cell cycle progression (38-41). This raises the possibility that phosphorylation at Ser131 may be a prerequisite for complete phosphorylation at Ser203 and concomitantly effective RASSF1A tumor suppression. Therefore, in the case of RASSF1A-p.133Ser, defective phosphorylation at Ser131 would lead to impaired RASSF1A function.

We have previously reported that ATM/RASSF1A promotes a robust p73 response in response to DNA damage (15). We now find that the minor allele variant, RASSF1A-p.133Ser, is unable to elicit a similar response (Figure 1D) and that this is likely through the impaired activation of MST/LATS signaling (Figure 1C-H). Apart from p73, we find that RASSF1A-p.133Ser also has an effect on the stabilization and activation of p53 (Figure 3). In line with previous reports highlighting RASSF1A/Daxx control of MDM2 activity (16), introduction of RASSF1A-p.133Ala leads to an increased stability of p53 in response to DNA damage (Figure 3B). However, this stabilization is attenuated in the presence of RASSF1A-p.133Ser, resulting in reduced expression of the p53 target gene p21 (Figure 3B). Additionally, the reduced p53 activation could also be due to the impaired ability of RASSF1A-p.133Ser to activate MST1/2 which would impact on both SIRT1 directed p53 deacetylation (17) and LATS1/2 mediated control of MDM2 activation (18, 19).
Taken together, these observations support the idea that \textit{RASSF1} c.397G>T results in the loss or impairment of a major tumor suppressor pathway in cells (4, 7, 10, 11, 24). In survival studies of soft tissue sarcoma patients, the minor allele associated with poorer survival with a significant relative risk of tumor-related death (50.5 vs 100.9 months, \(p=0.016\); \(RR=4.82, p=0.034\), Table 1) in males. Interestingly, the relative risk of tumor-related death was significantly higher in a subset of male STS patients that had been treated with radio/chemotherapy, suggesting an association of the SNP with therapeutic outcome following DNA-damaging treatment (\(RR=12.75, p=0.031\), Table 1). Though the sample size of patients carrying the minor allele is low, our results suggest that \textit{RASSF1} c.397G>T deserves further validation in larger cohorts to determine its usefulness as a prognostic indicator for survival in STS patients.

Our studies also demonstrate that male STS patients carrying at least one copy of the minor allele were diagnosed at a significantly earlier age than their wild-type counterparts (33.7 vs 55.9 years, \(p=0.0098\), Table 2) suggesting an association between the SNP and age of disease onset. Further validation of these associations in additional STS cohorts are necessary and it would be of interest to determine if these differences in the age of onset could ultimately translate into an overall increased risk for STS development in case-control studies designed to estimate risk. However, these findings are in agreement with a previous report suggesting an association between \textit{RASSF1} c.397G>T and early onset breast cancer in BRCA1/2 mutation carriers (24). Interestingly, no correlation was found in a subsequent study (42), indicating that additional factors may be required for the early onset phenotype in breast cancer. An intrinsic failure to respond to endogenous DNA damage stemming from cellular respiration,
environmental radiation or defective DNA repair (such as BRCA1/2 inactivation) leads to increased genomic instability and susceptibility to cancer (43). In the case of RASSF1 c.397G>T, a defective response to endogenous DNA damage and impaired p53/p73 tumor suppressive activity would precipitate tumorigenesis at an earlier age which would be exacerbated by higher levels of genomic instability in BRCA1/2 mutation carriers.

Interestingly, we and others had previously shown that inactivation of RASSF1A signaling by methylation associates with poorer survival in a number of tumor types including soft tissue sarcomas (7-12). In this report, we go on to provide evidence in a cohort of soft tissue sarcoma patients that this association is sex-specific, whereby hypermethylation of the RASSF1A promoter in tumors only associates with poorer survival in males. These observations are dramatically similar to our observations for the differing genotypes at the RASSF1 c.397G>T locus, whereby expression of the isoform with weaker RASSF1 signaling associates with poorer survival in males but not in females. We also only observed the association of RASSF1 c.397G>T with the onset of STS also in the male patients (Figure 5). A male sex bias had also previously been reported for the association of RASSF1 c.397G>T with lung adenocarcinoma risk in a Japanese population (25). Taken together, it is tempting to speculate that in some tissues and cancers, males could be more sensitive to reduction of RASSF1 signaling either by epigenetic silencing or the inherited predisposition of RASSF1A-p.133Ser.

The association of sex and risk has been observed in various cancer types (44-46) and is thought to be due to the effect of sex-specific hormones. The involvement of androgen receptors in STS has been reported (47), raising the possibility that male-specific hormones
might play a role in the ability of the SNP to affect the age of onset, survival and response to treatment in male patients. Interestingly, previous studies suggest that MST kinases are negatively regulated by androgen receptor (AR) signaling to AKT (48). As AR signaling is stimulated by the primarily male hormone androgen, MST signaling may be inherently weakened and therefore limiting in males and the pathway may be more sensitive to changes in upstream activating signals, such as RASSF1A activity. The failure to activate RASSF1A p.133Ser in response to DNA damage may combine with AR mediated inhibition of MST to ablate residual pathway activity in male patients, explaining the sex specific clinical manifestation of RASSF1A activity either through epigenetic silencing or the inherited predisposition of RASSF1A-p.133Ser. It would be of interest to further evaluate the potential of RASSF1c.397G>T as a biomarker for identifying male sub-groups that may benefit from cancer surveillance at an earlier age or alternative therapeutic interventions.

In conclusion, we have shown that RASSF1 c.397G>T has a profound effect on the ability of RASSF1A to respond to a DNA damage signal and to mediate its tumor suppressive effects. We demonstrate for the first time an association of RASSF1 c.397G>T with poor survival outcome and earlier onset of disease in male STS patients. Further validation of these associations in additional STS cohorts as well as additional tumor sites will assist in determining the extent of RASSF1 c.397G>T usefulness as a prognostic indicator for overall survival as well as therapeutic response, and as a biomarker for early screening for male patients more at risk for STS.
ACKNOWLEDGEMENTS

We thank Dr Karen Vousden for the FLAG-p53 and Dr Subham Basu for the FLAG-YAP expression plasmids. This work was funded by Cancer Research UK. Ludwig Institute for Cancer Research and the Medical Research Council (UK).
REFERENCES

LEGENDS

Figure 1. RASSF1A-p.133Ser is defective in response to DNA damage activation of the MST/LATS/p73 pathway

(A) U2OS cells were transfected with plasmids expressing HA-RASSF1A-p.133Ala or HA-RASSF1A p.133Ser and treated with cisplatin (20μM) and harvested 60 min later. Endogenous ATM was immunoprecipitated and subjected to Western Blot analysis with the indicated antibodies to detect the presence of RASSF1A isoforms.

(B) Immunoprecipitated FLAG-ATM was incubated with FLAG-RASSF1A proteins immunopurified from H1299 inducible cells in the presence of [γ32P]ATP. Reactions were resolved by SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membrane and visualized using a BioRad Phosphorimager. The membranes were subsequently probed with the indicated antibodies.

(C) H1299 tet-on inducible cells expressing control vector, FLAG-RASSF1A-p.133Ala or FLAG-RASSF1A p.133Ser were transfected with a plasmid expressing FLAG-YAP and then treated with 2μg/ml doxycycline for 24 hours, followed by serum starvation overnight (16 hours). The cells were then treated either with γ-irradiation (10Gy) or cisplatin (20μM) before being harvested 60 min later. Cell lysates were prepared and analyzed by immunoblotting with the indicated antibodies. YAP protein levels were quantitated by densitometry using ImageJ software. The relative intensity of the YAP protein bands in cells treated with ionizing radiation or cisplatin (compared to non DNA-damaged cells) are indicated beneath the blots.
(D) H1299 cells were transfected with pcDNA or plasmids expressing FLAG-RASSF1A-p.133Ala or FLAG-RASSF1A-p.133Ser and treated with cisplatin (20uM) for 24 hours. Cell lysates were prepared and analyzed by immunoblotting with the indicated antibodies.

(E) U2OS cells were transfected with pcDNA or plasmids expressing HA-RASSF1A-p.133Ala or HA-RASSF1A-p.133Ser together with FLAG-MST2. Following serum starvation overnight, FLAG-MST2 was immunoprecipitated and subjected to Western Blot analysis using a phosphospecific MST antibody that recognizes T180-phosphorylated MST2. Lysates were also blotted with the indicated antibodies.

(F) Endogenous MST2 was immunoprecipitated from control H1299 tet-on inducible cells or cells expressing FLAG-RASSF1A-p.133Ala or FLAG-RASSF1A-p.133Ser that had been serum starved overnight. The kinase activity of MST2 in the immunoprecipitates was determined by the in-gel kinase assay using MBP as a substrate. The immunoprecipitates and cell lysates were also subjected to Western Blot analysis with the indicated antibodies.

(G) U2OS cells were transfected with pcDNA or plasmids expressing FLAG-RASSF1A-p.133Ala or FLAG-RASSF1A-p.133Ser. Following serum starvation overnight, endogenous MST2 was immunoprecipitated and subjected to Western Blot analysis with the indicated antibodies. The membrane was subsequently stripped and reprobed with an antibody that detects LATS1 protein phosphorylated on Ser909, a known MST2 phosphorylation site.

(H) U2OS cells were transfected with pcDNA or plasmids expressing FLAG-RASSF1A-p.133Ala or FLAG-RASSF1A-p.133Ser. Following serum starvation overnight, endogenous p73 was immunoprecipitated and subjected to Western Blot analysis with the indicated antibodies.
**Figure 2. RASSF1A-p.133Ser is defective in sensitising cells to DNA damaging agents**

(A) H1299 cells were transfected with either pcDNA3 or plasmids expressing RASSF1A-p.133Ala or RASSF1A p.133Ser together with pBabe-Puro. Following puromycin selection, the cells were serum starved and treated with the indicated concentration of cisplatin. 48 hours later, cell viability was evaluated using the resazurin assay. Lysates were subjected to western blot analysis with the indicated antibodies to demonstrate expression levels of RASSF1A isoforms. Data are represented as mean +/- SEM.

(B) H1299 cells were transfected with either pcDNA3 or plasmids expressing RASSF1A-p.133Ala, RASSF1A-p.133Ser and RASSF1A-p.131Phe, serum starved and exposed to ionizing radiation (2Gy). Surviving colonies were counted after 14 days. Data are represented as mean +/- SEM.

**Figure 3. RASSF1A-p.133Ser restricts p53 activity in response to DNA damage**

(A) U2OS cells transfected with pcDNA or plasmids expressing Flag-RASSF1A-p.133Ala or Flag-RASSF1A-p.133Ser together with Flag-p53 were incubated with cycloheximide (50μg/ml) for the indicated times. Cell lysates were analysed by immunoblotting with the indicated antibodies.

(B) U2OS cells transfected with pcDNA or plasmids expressing Flag-RASSF1A-p.133Ala or Flag-RASSF1 A p.133Ser were incubated with adriamycin (0.2μg/ml) for the indicated times. Cell lysates were analysed by immunoblotting with the indicated antibodies.

(C) U2OS cells were transfected with pcDNA or plasmids expressing FLAG-RASSF1A-p.133Ala or FLAG-RASSF1A p.133Ser and treated with adriamycin (0.2μg/ml) for 24 hours. Cell lysates were
analysed by immunoblotting with the indicated antibodies. (D) U2OS tet-on inducible cells expressing control vector, FLAG-RASSF1A-p.133Ala or FLAG-RASSF1A p.133Ser were mock treated or treated with 2μg/ml doxycycline. The cell cycle profiles of these cells were determined 40 hours later by FACS analysis.

**Figure 4.** *RASSF1* c.397C>A and promoter methylation are associated with survival outcome in male STS patients.

(A) Kaplan-Meier curves showing tumor-specific survival rates for male and female STS patients stratified by genotype. (B) Cox multivariate regression curves showing tumor-specific survival rates for male and female STS patients stratified by genotype (total cohort, top panels; RCHT treated, bottom panels).

(C) Kaplan-Meier curves showing tumor-specific survival rates for male and female STS patients stratified by promoter methylation status. (D) Cox multivariate regression curves showing tumor-specific survival rates for male and female STS patients stratified by promoter methylation status. (E) Kaplan-Meier curves showing tumor specific survival rates for male and female STS patients homozygous for p.133Ala (G/G) stratified by promoter methylation status.

**Figure 5.** *RASSF1* c.397C>A is associated with age of onset of STS in males.

Graph showing cumulative number of individuals harboring at least one copy of the T-allele (diamonds) or G/G in genotype (squares) plotted against age at diagnosis for male (left) and female (right) STS patients.
Table 1. RASSF1 c.397C>A and promoter methylation are associated with poor survival in male STS patients.

Table of RASSF1A genotypes, promoter methylation status and overall survival time for total population and radio and/or chemotherapy treated population for male and female STS patients.

Table 2. RASSF1 c.397C>A is associated with early onset of STS in males.

Table of RASSF1A genotypes and age at diagnosis for male and female STS patients.
Yee et al. Table 1

### RASSF1 GENOTYPES, PROMOTER METHYLATION AND MEAN SURVIVAL TIME

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Note: G/G, homozygous for p.133Ala; G/T heterozygous for codon 133; T/T homozygous for p.133Ser
* Genotypes G/T and T/T grouped together, calculated against G/G
## RASSF1 Genotypes and Mean Age at Diagnosis of STS Patients

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*Mann Whitney Test, G/T+T/T vs G/G; two-tailed*
Yee et al. Figure 1

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IP: ATM

Lysate

ATM

RASSF1A

B

γ-IR

In vitro kinase

32P RASSF1A

FLAG (ATM)

FLAG (RASSF1A)

C

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Lysate

FLAG(YAP)

RASSF1A

GAPDH

D

Control

Cisplatin

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Lysate

PUMA

RASSF1A

TUBULIN

E

Flag-MST2

pcDNA

p.133A | p.133S

MST2

(pT180)

MST2

HA (R1A)

MST2

TUBULIN

F

H1299 tet-on

pcDNA

p.133A | p.133S

MST2

(pS909)

RASSF1A

TUBULIN

G

pcDNA

p.133A | p.133S

LATS1

MST2

RASSF1A

TUBULIN

H

pcDNA

p.133A | p.133S

YAP

p73

YAP

p73

RASSF1A

TUBULIN
A

H1299

% Viability

![Graph showing viability percentages for Control, RASSF1A-p.133Ala, and RASSF1A-p.133Ser across different concentrations of Cisplatin.]

B

H1299

Clonogenic Survival

![Graph showing clonogenic survival with p < 0.05 for different treatments.]

RASSF1A and TUBULIN protein levels in Lysate samples:

- Con
- p.133Ala
- p.133Ser
- p.133Phe
Yee et al. Figure 3

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**C**

- Control
- Adriamycin

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Yee et al. Figure 4

A

MALE

FEMALE

Cumulative Survival

\[ p=0.016 \quad p=0.778 \]

Time (months)

G/T

G/G

0

20

40

60

80

100

120

140

B

MALE

FEMALE

ALL

PATIENTS

Cumulative Survival

\[ p=0.034 \quad RR=4.82 \]

\[ p=0.722 \quad RR=0.82 \]

Time (months)

G/T + T/T

G/G

0

20

40

60

80

100

120

RCHT

TREATED

PATIENTS

Cumulative Survival

\[ p=0.031 \quad RR=12.75 \]

\[ p=0.627 \quad RR=1.48 \]

Time (months)

G/T + T/T

G/G

C

MALE

FEMALE

Cumulative Survival

\[ p=0.002 \quad p=0.368 \]

Time (months)

NON-METHYLATED

METHYLATED

D

MALE

FEMALE

Cumulative Survival

\[ p=0.002 \quad RR=9.69 \]

\[ p=0.664 \quad RR=0.75 \]

Time (months)

NON-METHYLATED

METHYLATED

E

MALE

FEMALE

Cumulative Survival

\[ p=0.07 \quad p=0.368 \]

Time (months)

NON-METHYLATED

METHYLATED
Yee et al. Figure 5

**MALES**

- **Age of diagnosis vs. Percent incidence**
- **T/T + T/G**
- **G/G**
- **P = 0.008**
- **22.2 years difference**

**FEMALES**

- **Age of diagnosis vs. Percent incidence**
- **T/T + T/G**
- **G/G**
- **P = 0.002**
- **1.3 years difference**
A RASSF1A polymorphism restricts p53/p73 activation and associates with poor survival and accelerated age of onset of soft tissue sarcoma.


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