Review

Epigenetic inactivation of the Ras-association domain family 1 (*RASSF1A*) gene and its function in human carcinogenesis

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Summary. The Ras GTPases are a superfamily of molecular switches that regulate cellular proliferation and apoptosis in response to extra-cellular signals. The regulation of these pathways depends on the interaction of the GTPases with specific effectors. Recently, we have cloned and characterized a novel gene encoding a putative Ras effector: the Ras-association domain family 1 (*RASSF1*) gene. The *RASSF1* gene is located in the chromosomal segment of 3p21.3. The high allelic loss in a variety of cancers suggested a crucial role of this region in tumorigenesis. At least two forms of *RASSF1* are present in normal human cells. The *RASSF1A* isoform is highly epigenetically inactivated in lung, breast, ovarian, kidney, prostate, thyroid and several other carcinomas. Re-expression of *RASSF1A* reduced the growth of human cancer cells supporting a role for *RASSF1* as a tumor suppressor gene. *RASSF1A* inactivation and K-ras activation are mutually exclusive events in the development of certain carcinomas. This observation could further pinpoint the function of *RASSF1A* as a negative effector of Ras in a pro-apoptotic signaling pathway. In malignant mesothelioma and gastric cancer *RASSF1A* methylation is associated with virus infection of SV40 and EBV, respectively, and suggests a causal relationship between viral infection and progressive *RASSF1A* methylation in carcinogenesis. Furthermore, a significant correlation between *RASSF1A* methylation and impaired lung cancer patient survival was reported, and *RASSF1A* silencing was correlated with several parameters of poor prognosis and advanced tumor stage (e.g. poor differentiation, aggressiveness, and invasion). Thus, *RASSF1A* methylation could serve as a useful marker for the prognosis of cancer patients and could become important in early detection of cancer.

Key words: *RASSF1*, Ras, tumor suppressor gene, cancer, methylation

Introduction

The proto-oncogene Ras plays a crucial role in the regulation of cell proliferation and cell death in response to external signals. Aberrant Ras function is involved in the development of cancer and Ras is frequently mutated in several types of tumors (Bos, 1989; Barbacid, 1990). Mutation of Ras (mainly at codon 12) leads to the transformation of Ras to an oncogene and results in constitutively activated signaling (Crespo and Leon, 2000). The Ras superfamily of small GTPases transmits signals from receptor tyrosine kinases to the nucleus and regulates cell growth, survival, and differentiation (Campbell et al., 1998; Khosravi-Far et al., 1998; Marshall, 1999; Bar-Sagi, 2001). Ras proteins function as switches with two different conformations: active when GTP is bound and the inactive GDP-bound state. The Ras activity is inhibited through GTPase-activating proteins (GAP) by increasing the rate of hydrolysis of bound GTP. Guanine nucleotide exchange factors (GEF) or dissociation stimulators (GDS) positively regulate the GTP binding activity of Ras. The activated Ras acts in well-studied pathways by regulating the cellular

Abbreviations: RASSF, Ras-association domain family; NORE, Novel Ras effector; LOH, Loss of heterozygosity; RA domain, RalGDS/AF6 Ras-association domain; aa, amino acid; 5-aza-CdR, 5-aza-2'-deoxycytidine; SV40, simian virus 40; EBV, Epstein-Barr virus; ATM, ataxia telangiectasia mutated; DAG, diacylglycerol; C1, protein kinase C conserved region; SCLC, small cell lung carcinoma; NSCLC, non-small cell lung carcinoma; MM, malignant mesothelioma; NPC, nasopharyngeal carcinoma; CRC, colorectal carcinoma; TC, thyroid carcinoma

response through distinct Ras effectors and their complex cascades in signaling transduction (Fig. 1) (Katz and McCormick, 1997; Khosravi-Far et al., 1998; Yamamoto et al., 1999; Reuther and Der, 2000).

The best-characterized signal transduction pathway of Ras is by the Raf serine/threonine kinases (Leevers and Marshall, 1992; Kolch, 2000). Activated Raf phosphorylates MEK (MAPK/ERK kinase). Subsequently, the activated MEK phosphorylates the MAPK (mitogen-activated protein kinase). As a result the activated MAPK is translocated into the nucleus, where it phosphorylates a set of transcription factors. For example, the activation of Elk-1 leads to the transcription of Fos, which forms together with the MAPK-activated Jun the activation protein 1 (AP-1). AP-1 was shown to induce cyclin D1 and therefore to stimulate proliferation (Shaulian and Karin, 2001).

The second cascade of Ras-activated signaling is by the lipid kinase, phosphatidylinositol 3-kinase (PI3-K) and prevents cells from undergoing apoptosis (Downward, 1998; Datta et al., 1999). PI3-K converts

Fig. 1. Ras-signaling pathway. Growth factor-mediated response of the receptor tyrosine kinase (RTK) activates Ras-GTP. Ras regulates several signaling pathways. The MAP-kinase pathway (Raf, MEK, and MAPK/ERK) activates cellular proliferation. *RASSF1A* blocks cell-cycle progression and inhibits cyclin D1 accumulation. Ras inhibits apoptosis by the pathway of the phosphatidylinositol 3 kinase (PI3-K) and stimulates the activity of the protein kinase B Akt (Akt/PKB). Subsequently, Akt inhibits apoptosis induced by members of the Bcl-family (BAD). Additionally, Ras regulates a pro-apoptotic pathway by binding to the Ras effectors NORE1 and *RASSF1A* and activation of the apoptotic protein kinase MST1.

lipids, which can stimulate the activity of the protein kinase B Akt. Subsequently, Akt neutralizes BAD by phosphorylation. BAD is a pro-apoptotic member of the Bcl-family (Korsmeyer, 1999). Activated BAD proteins form heterodimers with the anti-apoptotic BCL-2 and BCL-XL, which is a crucial signal for programmed cell death. Inactivating BAD enables BCL to promote the cell survival by blocking the release of mitochondrial cytochrome c and therefore inhibiting caspase activation. Anti-apoptotic activity of Akt also includes the phosphorylation of pro-caspase-9, the upregulation of the NF-κB transcription factor and the regulation of forkhead family members (Cardone et al., 1998; Kops et al., 1999; Romashkova and Makarov, 1999).

Raf and the PI3-K are well-characterized Ras effectors, which interact with Ras by Ras-binding domains (RBD and PI3K_rbd, respectively). A third group of Ras effectors shares a conserved motif, the RalGDS/AF6 Ras-association (RA) domain, which does not have a highly significant similarity with the RBD and PI3K_rbd (Ponting and Benjamin, 1996; Yamamoto et al., 1999). The identification of sequence homologies between the two Ras effectors, Ral guanosine nucleotide-exchange factor (RalGDS) and the ALL-1 fusion partner from chromosome 6 (AF-6), defined the RA domain (Ponting and Benjamin, 1996). RalGDS contributes to the Ras-induced transformation and AF-6 is involved in the regulation of cell adhesion (Katz and McCormick, 1997; Wolthuis and Bos, 1999; Yamamoto et al., 1999). Recently new genes encoding the RA domain have been identified: the Ras-association domain family (Vavvas et al., 1998; Dammann et al., 2000; Tommasi et al., 2002). RASSF1, RASSF3 and NORE1 (RASSF5) are potential Ras effectors (Fig. 2). RASSF1A, the major isoform of RASSF1, blocks cell-cycle progression and inhibits cyclin D1 accumulation (Fig. 1) (Shivakumar et al., 2002). Additionally, Ras regulates a pro-apoptotic pathway by the binding of the Ras effector NORE1 and RASSF1A to the apoptotic protein kinase MST1 (Fig. 1) (Khokhlatchev et al., 2002). The alteration and function of *RASSF1A* is the subject of this review.
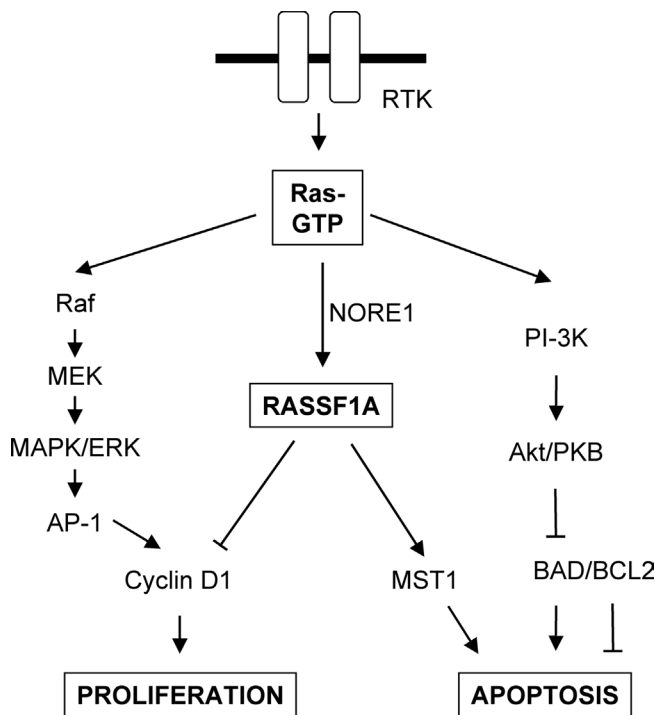
Identification and characterization of RASSF1

Genetic factors may not contribute greatly to the development of human lung cancer. Thus, responsible genes have not been mapped in most of the chromosomal deletion regions. Instead, a considerable effort is being devoted to defining the most frequently occurring regions of chromosomal deletions. Of particular importance are genes located within regions in which both alleles are lost in cancers (homozygous deletions). The size of a homozygously-deleted region is often much smaller than that of a hemizygously-deleted region. In the past, several tumor suppressor genes, including *RB*, *p16*, *SMAD4* and *PTEN*, have been isolated and cloned from regions homozygously deleted in cancer. Homozygous deletions at segment 3p21.3

have been described in several cancer cell lines and in primary lung tumors (Killary et al., 1992; Yamakawa et al., 1993; Wei et al., 1996; Kok et al., 1997; Todd et al., 1997; Wistuba et al., 2000). The region of minimum homozygous deletion was narrowed to 120 kb using several lung cancer cell lines and a breast cancer cell line (Sekido et al., 1998).

Recently, we and others have cloned the *RASSF1* gene from the common homozygous deletion area at 3p21.3 (Dammann et al., 2000; Burbee et al., 2001). RASSF1C was isolated through the interaction with the human DNA excision repair protein XPA in a yeast two-hybrid screen (Dammann et al., 2000). The 1.7 kb cDNA matched the sequences of the minimum homozygous deletion region of 120 kb (Sekido et al., 1998) that may

contain the tumor suppressor gene at 3p21.3. The C-terminus of RASSF1C shows high homology (ca. 55% identity) to the murine Ras-effector protein Nore1 (Vavvas et al., 1998) and encodes a Ras-association domain (Fig. 2). Therefore, the gene has been termed Ras-association domain family 1 gene (Dammann et al., 2000). Homology searches and additional cDNA screenings revealed the presence of three alternatively spliced transcripts: *RASSF1A*, *RASSF1B* and *RASSF1C* (Dammann et al., 2000). The two major forms *RASSF1A* and *RASSF1C* are transcribed from two distinct CpG island promoters, which are approximately 3.5 kb apart (Fig. 2). All transcripts have four common exons (3 to 6) at their 3' end (Fig. 2). These exons encode the RA domain (R194 to S288) (Ponting and Benjamin, 1996). *RASSF1A* has two 5' exons, designated 1α and 2αß. The cDNA is 1.9 kb long and contains an ORF of 340 amino acids (aas) with a calculated MW of 38.8 kDa. The N-terminus (H52 to C101) of *RASSF1A* has high homology to a cystein-rich diacyl-glycerol/phorbol ester-binding (DAG) domain, also known as the protein kinase C conserved region (C1), which contains a central C1 zinc finger (Newton, 1995). *RASSF1A* is expressed in all normal tissues tested by Northern blot analysis, but was missing in several cancer cell lines (Dammann et al., 2000). The minor transcript RASSF1B has the same exon 2αß but the first exon 1ß is different from that of transcript A (Fig. 2) and is mainly expressed in tissue containing cells from the haematopoetic system. Most likely, the 1.7 kb cDNA of *RASSF1B* encodes only the RA domain and in the murine Rassf1 locus exon 1ß is completely missing (see GenBank accession number AF333027). Transcript *RASSF1C* is 1.7 kb long and transcription initiates in exon 2γ located at the CpG island C (Fig. 2). The cDNA encodes a 270 aas protein with an MW of 31.2 kDa. The protein sequence translated from the first exon 2γ has no significant similarity to any known protein. *RASSF1C* is transcribed in all normal tissue and cancer cells tested (Dammann et al., 2000). The aa sequence W125 to K138 (WETPDLSQAEIEQK) of *RASSF1A* matches a putative ATM kinase phosphorylation consensus motif and in a peptide with this sequence serine is effectively phosphorylated in vitro (Kim et al., 1999). In NORE1 and RASSF3 this ATM consensus site, which contains a minimal SQ target is missing (Fig. 2) (Tommasi et al., 2002).

RASSF1 was cloned independently by another group, which isolated four additional isoforms (Burbee et al., 2001). These four forms: *RASSF1D*, *RASSF1E*, *RASSF1F* and *RASSF1G* are splicing variants of *RASSF1A* and are transcribed from the CpG island A promoter (Fig.2). RASSF1D and RASSF1E are heart-specific and pancreas-specific forms, respectively. RASSF1D encodes four additional aas (LSAD) 5' of exon 2αß. RASSF1E has four additional aas (PILQ) 3' of exon 2αß. The RASSF1F transcript skips exon 2αß and encodes a truncated peptide of 92 aas ending within the DAG-binding domain (Burbee et al., 2001) and
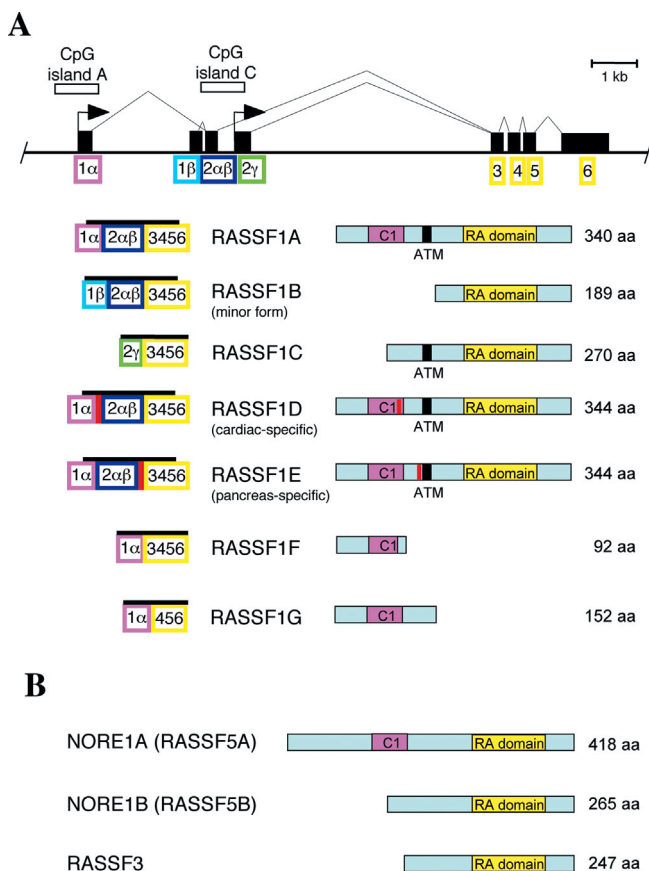

Fig. 2. The Ras-association domain family. **A.** Map of the *RASSF1* gene at 3p21.3 encoding different isoforms. The two promoters of *RASSF1*(arrows) are located in CpG islands (open squares). Seven different isoforms (*RASSF1A* to G) are made by alternative promoter usage and RNA splicing of the exons (black boxes). The red boxes indicate additional sequences in the cardiac- and pancreas-specific isoforms. The encoded protein length is indicated in amino acid (aa) and domains are marked as: DAG, diacylglycerol/phorbol ester binding domain; RA, RalGDS/AF6 Ras-association domain; and ATM, putative ATM phosphorylation site consensus sequence. **B.** The protein sequence of two isoforms of NORE1 (RASSF5A and RASSF5B) and RASSF3 are indicated.

RASSF1G misses exon 2αß and exon 3 and has a predicted size of 152 aas (Fig. 2). Only the *RASSF1F* transcript is frequently detected by RTPCR, but the biological function of these additional transcripts is unknown (Burbee et al., 2001). However, all *RASSF1* forms, which are transcribed from CpG islands A, are frequently missing in a variety of tumors as a result of epigenetic inactivation of the *RASSF1A* promoter.

Mutational analysis of RASSF1A in tumors

In general, both alleles of a tumor suppressor gene need to be inactivated by genetic alterations such as chromosomal deletions or loss-of-function mutations in the coding region of a gene (Knudson, 1971). All exons of the *RASSF1* gene were intensively sequenced for mutational events, but so far only very few mutations have been identified (Table 1). We have analyzed 58 lung carcinomas for *RASSF1A* mutations (Dammann et al., 2000). Two missense mutations were identified: ATT(Ile135) to ACT(Thr135); and GCC(Ala336) to ACC(Thr336). However, it is unclear if these alterations represent rare polymorphisms (Table 1). Two additional changes did turn out to be polymorphisms. The International Lung Cancer Chromosome 3p21.3 Tumor Suppressor Gene Consortium has analyzed the sequence of 114 lung carcinomas for *RASSF1* mutations (Lerman and Minna, 2000) and several polymorphisms were identified, but no somatic mutations (Burbee et al., 2001). In 40 breast carcinomas several polymorphisms

were identified (Agathanggelou et al., 2001). No somatic mutations were found in an additional 20 primary breast cancers (Dammann et al., 2001a) and no inactivating mutations were detected in 10 phaeochromocytomas (Astuti et al., 2001). Recently, 29 nasopharyngeal carcinomas were screened for *RASSF1A* mutations (Lo et al., 2001). Several additional polymorphisms were detected. Interestingly, a missense mutation CGC(Arg201) to CAC(His201) and a frameshift mutation were identified (Table 1). Additional changes were detected in kidney carcinoma cell lines (Dreijerink et al., 2001). In summary, only two confirmed somatic mutations were found in more than 200 samples (Table 1).

Seven of the 21 characterized changes are silent (Table 1). Five polymorphisms are located in the DAG-binding site, four polymorphisms in the ATM kinase consensus motif and four in the RA domain (Table 1). It will be interesting to analyze whether individuals with certain polymorphisms are more susceptible for lung cancer. Recently Shivakumar et al. (2002) have shown that two single nucleotide polymorphisms located in the putative ATM kinase phosphorylation site of *RASSF1A* (S131F and A133S) encode proteins that fail to block cell-cycle progression. The mutation frequency of other genes located within the 3p21.3 homozygous deletion area was found to be less than 8% in lung tumors (Lerman and Minna, 2000). This strengthens the assumption that the putative 3p21.3 tumor suppressor gene is inactivated by mechanisms other than mutations

Table 1. Mutational analysis of the *RASSF1A* gene in human cancer[a].

CHANGE	CODON[b]	CODON CHANGES	EXON	DOMAIN[d]
Alteration[c]	6	GAG (Glu) to Asp	1α	
Common polymorphism	21	AAG(Lys) to CAG(Gln)	1α	
Common polymorphism	28	CGT(Arg) to CGA(Arg) silent	1α	
Polymorphism	49	GGC(Gly) to GGT(Gly) silent	1α	
Common polymorphism	53	CGC(Arg) to TGC(Cys)	1α	DAG
Polymorphism	53	CGC(Arg) to CGT(Arg) silent	1α	DAG
Polymorphism	56	CCC(Pro) to CCT(Pro) silent	1α	DAG
Polymorphism	57	GCG(Ala) to GCA(Ala) silent	1α	DAG
Polymorphism	60	GCC(Ala) to ACC(Thr)	1α	DAG
Common polymorphism	129	GAC(Asp) to GAG(Glu)	3	ATM
Polymorphism	131	TCT(Ser) to TTT(Phe)	3	ATM
Common polymorphism	133	GCT(Ala) to TCT(Ser)	3	ATM
Alteration[c]	135	ATT(Ile) to ACT(Thr)	3	ATM
Missense mutation	201	CGC(Arg) to CAC(His)	4	RA
Polymorphism	214	CTG(Leu) to CTA(Leu) silent	4	RA
Polymorphism	236	GTG(Val) to GTA(Val) silent	4	RA
Polymorphism	246	GAG(Glu) to AAG(Lys)	4	RA
Common polymorphism	257	CGG(Arg) to CAG(Gln)	5	RA
Frameshift mutation	277	1 bp deletion at nt 829	5	RA
Polymorphism	325	TAT(Tyr) to TGT(Cys)	6	
Alteration[c]	336	GCC(Ala) to ACC (Thr)	6	

[a]: see references (Dammann et al., 2000; Agathanggelou et al., 2001; Burbee et al., 2001; Dreijerink et al., 2001; Lo et al., 2001; Shivakumar et al., 2002). [b]: The codons are numbered using *RASSF1A* as a reference. [c]: This alteration represents a mutation or rare polymorphism. [d]: ATM: putative ATM phosphorylation site consensus sequence; DAG: diacylglycerol/phorbol ester-binding domain; RA: RalGDS/AF6 Ras-association domain.

of the coding sequence.

The RASSF1A CpG island promoter is epigenetically silenced in primary tumors

An alternative mechanism of gene inactivation in human cancer is epigenetic inactivation of tumor suppressor genes (Jones and Laird, 1999). In particular, transcriptional silencing by hypermethylation of CpG sequences in the CpG island promoter regions of genes is becoming recognized as a common mechanism of gene inactivation (Baylin and Herman, 2000). Recent studies have demonstrated that the CpG islands of the *RB*, *p16*, *VHL*, *APC*, *MLH1*, and *BRCA1* genes are frequently methylated in a variety of human cancers, but are largely methylation-free in the corresponding normal tissues (Baylin et al., 1998; Jones and Baylin, 2002). It is remarkable that as many, if not more, tumor suppressor genes are inactivated by promoter hypermethylation as they are by coding-region mutations. Therefore, epigenetic silencing of tumor suppressor genes plays a key role in human carcinogenesis.

RASSF1 expression and the methylation pattern of the two CpG island promoters of *RASSF1A* and *RASSF1C* have been analyzed in a variety of cancer cell lines and primary tumors. In all normal cells both forms, *RASSF1A* and C, are highly expressed (Dammann et al., 2000). *RASSF1A* expression is missing in several cancer cell lines including non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), breast carcinoma, nasopharyngeal carcinoma, renal cell carcinoma, and thyroid carcinoma (Dammann et al., 2000, 2001a,b; Burbee et al., 2001; Dreijerink et al., 2001; Lo et al., 2001; Schagdarsurengin et al., 2002). In contrast, *RASSF1C* was expressed in all analyzed samples without homozygous deletion of 3p21.3 (Dammann et al., 2000, 2001a,b; Burbee et al., 2001; Schagdarsurengin et al., 2002). In primary tumor expression analysis, is more difficult because normal cells are always present. Nevertheless, in primary breast carcinomas and lung adenocarcinomas *RASSF1A* expression is highly reduced (Burbee et al., 2001; Dammann et al., 2001a). Loss of expression was correlated with hypermethylation of the CpG island of the *RASSF1A* promoter (Dammann et al., 2000; Agathanggelou et al., 2001). In addition, reversion of the epigenetic silencing by treatment with a DNA methylation inhibitor, 5-aza-2'-deoxycytidine (5-aza-CdR), leads to re-expression of *RASSF1A* in various cancer cell lines (Astuti et al., 2001; Burbee et al., 2001; Byun et al., 2001; Dammann et al., 2001a; Lo et al., 2001; Schagdarsurengin et al., 2002; Toyooka et al., 2002).

The *RASSF1A* inactivation in primary tumors was carefully investigated by methylation analysis of the CpG island A promoter. Table 2 summarizes the methylation status of the *RASSF1A* promoter in different primary tumors. In small cell lung carcinoma (SCLC) the percentage of methylation of the *RASSF1A* CpG island is very high and ranges from 70% to 80%

(Agathanggelou et al., 2001; Burbee et al., 2001; Dammann et al., 2001b). Since LOH at 3p21.3 occurs in almost 100% of all SCLC (Kok et al., 1997; Girard et al., 2000; Lindblad-Toh et al., 2000; Wistuba et al., 2000), the remaining allele of *RASSF1A* is silenced by methylation (Burbee et al., 2001).

LOH at 3p21 has been reported to be frequent in non-small cell lung cancer (NSCLC) (Kok et al., 1997). Methylation of *RASSF1A* appears to be common in NSCLC (Table 2) and inactivation of *RASSF1A* was found in 30 to 38% of primary NSCLC, but in none of the nonmalignant lung tissue (Dammann et al., 2000; Agathanggelou et al., 2001; Burbee et al., 2001). Interestingly, Burbee et al. (2001) reported that in resected NSCLC, *RASSF1A* promoter hypermethylation was associated with impaired survival (P=0.046). Recently, Tomizawa et al. (2002) reported that in stage I lung adenocarcinoma RASS1A methylation correlates with adverse survival of cancer patients (P=0.0368 and P=0.032 by univariant and multivariant analysis, respectively). Additionally, *RASSF1A* methylation was detected in 32% of adenocarcinoma and was detected more frequently in tumors with vascular invasion and pleural involvement. *RASSF1A* inactivation was more frequently observed in poorly-differentiated tumors than in well- (P=0.00059) or moderately- (P=0.0835) differentiated adenocarcinoma (Tomizawa et al., 2002). Recently, Belinsky et al. (2002) analyzed the aberrant promoter methylation of *RASSF1A* and other tumor suppressor genes (e.g. *p16*, *MGMT* and *DAP-Kinase*) in bronchial epithelium and sputum from current and former smokers. No *RASSF1A* inactivation was detected in the bronchial epithelium and was only seen in 2 out of 66 (3%) of sputum controls (Belinsky et al., 2002). This result suggests that inactivation of *RASSF1A* could be a later event in malignant transformation of bronchial epithelium.

We and others have analyzed *RASSF1A* inactivation in primary breast carcinoma (Table 2). Two reports indicate 49% and 62% of *RASSF1A* inactivation. However, another study detected only 9% of hypermethylation (Agathanggelou et al., 2001; Burbee et al., 2001; Dammann et al., 2001a). Lehmann et al. (2002) assessed aberrant *RASSF1A* promoter methylation during breast cancer development. *RASSF1A* was almost completely methylated in 56% of ductal breast carcinoma. *RASSF1A* inactivation was also demonstrated in epithelial hyperplasia, intraductal papillomas, but was not detected in lymphocytes, stromal tissue, normal breast epithelium, lactating breast tissue or apocrine metaplasia.

RASSF1A epigenetic inactivation was investigated in tumors of the female genital tract (Table 2). One ovarian cancer suppression region overlapped with the locus at 3p21.3 (Fullwood et al., 1999). Yoon et al. (2001) demonstrated 40% of *RASSF1A* methylation in ovarian carcinoma. Another group reported 10% of inactivation in ovarian carcinoma, although no methylation was detected in cervical carcinoma (Agathanggelou et al.,

Table 2. Methylation analysis of the *RASSF1A* gene in human tumors.

TUMOR TYPE	PERCENT OF METHYLATION IN PRIMARY TUMORS	REFERENCE	ADDITIONAL OBSERVATIONS
Non-small cell lung cancer	38% (22/58)	Dammann et al., 2000	Exogenous expression of *RASSF1A* inhibited growth of lung cancer cells in vitro and in vivo
	34% (14/41)	Agathanggelou et al., 2001	
	30% (32/107)	Burbee et al., 2001	Methylation of *RASSF1A* was associated with impaired patient survival (P=0.046)
	32% (35/110)	Tomizawa et al., 2002	*RASSF1A* methylation correlated with adverse survival of lung adenocarcinoma patients
Small cell lung cancer	79% (22/28)	Dammann et al., 2001b	
	72% (21/29)	Agathanggelou et al., 2001	
Breast cancer	62% (28/45)	Dammann et al., 2001a	*RASSF1A* was reexpressed after treatment with 5-aza-CdR in breast cancer cell lines
	9% (4/44)	Agathanggelou et al., 2001	
	49%(19/39)	Burbee et al., 2001	
	56% (20/36)	Lehmann et al., 2002	*RASSF1A* methylation was detected in epithelial hyperplasia, but not in normal tissue
Ovarian cancer	10% (2/21)	Agathanggelou et al., 2001	
	40% (8/20)	Yoon et al., 2001	
Cervical cancer	0% (0/22)	Agathanggelou et al., 2001	
Malignant mesothelioma	32% (21/66)	Toyooka et al., 2001	Inactivation of *RASSF1A* was correlated with the presence of SV40 in mesothelioma (P=0.022)
Phaeochromo-cytoma	22% (5/23)	Astuti et al., 2001	
	50% (13/26)	Dammann et al.,a	
Neuroblastoma	55% (37/67)	Astuti et al., 2001	*RASSF1A* was reexpressed after treatment with 5-aza-CdR in neuroblastoma cell lines
Renal cell carcinoma	56% (18/32)	Yoon et al., 2001	
	91% (39/43)	Dreijerink et al., 2001	Forced expression of *RASSF1A* in a renal carcinoma cell line suppressed growth in vitro
CC-RCC	23% (32/138)	Morrissey et al., 2001	*RASSF1A* was reexpressed after treatment with 5-aza-CdR in cancer cell lines
papillary RCC	44% (12/27)	Morrissey et al., 2001	
Nasopharyngeal cancer	67%(14/21)	Lo et al., 2001	No significant correlation between methylation of *RASSF1A* and clinical parameters
	50% (8/16)	Tong et al., 2002	*RASSF1A* methylation was detected in 39% of EBV associated NP brushing samples
Head and neck cancer	8% (6/80)	Hasegawa et al., 2002	
	17% (4/24)	Hogg et al., 2002	*RASSF1A* methylation was higher in poorly-differentiated HNSCC (P=0.0048)
Gastric cancer	43% (39/90)	Byun et al., 2001	Down-regulation of *RASSF1A* was correlated with advanced tumor stage
	67% (14/21)	Kang et al., 2002	Epstein-Barr virus-positive carcinoma
	4% (2/56)	Kang et al., 2002	Epstein-Barr virus-negative carcinoma
Bladder cancer	60% (33/55)	Lee et al., 2001	Inactivation of *RASSF1A* was correlated with advanced tumor stage
	35% (34/98)	Maruyama et al., 2001	*RASSF1A* methylation correlated with parameters of poor prognosis
Prostate cancer	53% (54/101)	Maruyama et al., 2002	*RASSF1A* methylation was correlated with clinicopathological features of poor prognosis
	100% (11/11)	Kuzmin et al., 2002	Reintroduction of *RASSF1A* suppressed the growth of a prostate cancer cell line in vitro
	71% (37/52)	Liu et al., 2002	*RASSF1A* methylation frequency was higher in more aggressive tumors (P=0.032)
Colon cancer	12% (3/26)	Yoon et al., 2001	
	20% (45/222)	van Engeland et al., 2002	*RASSF1A* methylation occurred predominantly in K-ras wild-type colorectal carcinomas (P=0.023)
	45% (13/29)	Wagner et al., 2002	*RASSF1A* was reexpressed after treatment with 5-aza-CdR in a colon cancer cell line
Thyroid cancer	71% (27/38)	Schagdarsurengin et al., 2002	*RASSF1A* methylation was more frequent in more aggressive thyroid carcinomas
Pediatric tumors (10 types)	40% (70/175)	Harada et al., 2002	e.g. 42% in Wilms tumor, 88% in medulloblastoma, 59% in retinoblastoma
Wilms tumor	73% (22/30)	Ehrlich et al., 2002	
	54% (21/39)	Wagner et al., 2002	

a: unpublished data

2001).

Aberrant methylation of *RASSF1A* was detected in 32% of malignant mesothelioma (MM) and was more common in epithelial MM than in sarcomatous/mixed MM (Toyooka et al., 2001). Interestingly, the frequency of *RASSF1A* inactivation was significantly higher in simian virus 40 (SV40) sequence positive MM than in negative MM and this demonstrates a relationship between SV40 infection and methylation (Toyooka et al., 2001). In a second study Toyooka et al. (2002) studied the role of SV40 infection for de novo methylation of CpG islands in normal human mesothelial cell lines. In early passages of infected cells no methylation was detected, but in late passages de novo methylation and loss of expression of *RASSF1A* was observed. This result suggests a causal relationship between SV40 infection and progressive *RASSF1A* silencing in the carcinogenesis of mesothelioma.

RASSF1A promoter region was found hyper-methylated in 22% of sporadic phaeochromocytomas and in 55% of neuroblastomas (Astuti et al., 2001). In two neuroblastoma cell lines methylation of *RASSF1A* correlated with loss of expression and was restored after treatment with a 5-aza-CdR (Astuti et al., 2001). Our own unpublished data showed 50% of *RASSF1A* methylation in phaeochromocytomas (Table 2).

In clear-cell renal-cell carcinoma (CC-RCC), the chromosomal arm that is most commonly affected by LOH is 3p and a major role of 3p21 has been suggested (van den Berg and Buys, 1997; Clifford et al., 1998; Kok et al., 2000; Martinez et al., 2000). In a sizable proportion of sporadic kidney carcinomas, VHL mutations are absent and an involvement of a gene on 3p21 is suspected. *RASSF1A* promoter methylation in renal-cell carcinoma (RCC) was 23% to 91% (Dreijerink et al., 2001; Morrissey et al., 2001; Yoon et al., 2001). Hypermethylation of the *RASSF1A* CpG island was similar in VHL-associated CC-RCC and in CC-RCC without VHL inactivation (Dreijerink et al., 2001; Morrissey et al., 2001). *RASSF1A* transcription was reactivated after treatment with 5-aza-CdR in renal-cancer cell lines (Dreijerink et al., 2001; Morrissey et al., 2001).

In nasopharyngeal carcinomas (NPC) of southern China the methylation frequency of the *RASSF1A* CpG island is 67% to 83% (Lo et al., 2001; Kwong et al., 2002). However, Kwong et al. (2002) found no significant correlation between the inactivation of *RASSF1A* and clinical parameters including stage, development of local regional recurrence, distant metastasis, or survival. In NPC 71% of tumors showed LOH of 3p21 (Lo et al., 2001). Epstein-Barr virus (EBV) is a ubiquitous herpes virus often associated with NPC and lymphoma. In nasopharyngeal biopsy and brushes from EBV-related NPC patients, *RASSF1A* methylation was detected in 50% and 39% of samples, respectively (Table 2) and EBV DNA was present in 96% (27 out of 28) cases (Tong et al., 2002). NPC is only one form of head and neck cancer. In squamous cell cancer of the head and neck (HNSCC) *RASSF1A* promoter methylation was 8% (Hasegawa et al., 2002) and Hogg et al. (2002) reported a *RASSF1A* methylation frequency of 17% in HNSCC and 66% of LOH for 3p21.3 marker in this carcinoma. Furthermore, they detected *RASSF1A* methylation to be significantly higher in poorly-differentiated than in moderate to well-differentiated HNSCC (P=0.0048).

RASSF1A methylation was investigated in 90 primary gastric carcinomas and was detected in 43% of cases and 60% of gastric carcinoma cell lines (Table 2) (Byun et al., 2001). CpG island methylation was correlated with abnormally low levels of *RASSF1A* expression in gastric carcinomas compared to normal gastric tissues and expression was restored by treatment with 5-aza-CdR in cancer cell lines. Silencing of *RASSF1A* was significantly higher in advanced tumors (63%) compared with early-stage tumors (26%; P<0.0001) and more frequent in poorly-differentiated tumors (62%) than well- (33%) or moderately-differentiated tumors (31%; P=0.01) (Byun et al., 2001). However, *RASSF1A* alteration showed no association with histological types of tumor. Kang et al. (2002) investigated *RASSF1A* methylation in Epstein-Barr virus (EBV)-related gastric carcinoma. Interestingly, methylation frequency of EBV-positive gastric carcinoma was significantly higher for more than 10 genes tested (e.g. *APC*, *p16*, *DAP-Kinase*, *PTEN*, *GSTP1* and *RASSF1A*). In 67% of EBV-positive tumors and 4% of EBV-negative gastric carcinomas aberrant methylation was detected (P<0.001; Table 2). Therefore, EBV-positive gastric carcinoma constitutes CpG island methylator-positive gastric carcinoma and suggests that methylation might be an important mechanism in EBV-related gastric carcinogenesis (Kang et al., 2002)

Two different groups investigated the *RASSF1A* inactivation in primary bladder cancers (Table 2). Lee et al. (2001) demonstrated that *RASSF1A* was methylated in 60% of tumors, in 80% of bladder cancer cell lines and that expression in cell lines was restored by treatment with 5-aza-CdR. In addition, altered expression of *RASSF1A* was observed in 79% of muscle–invasive tumors (T2-T4) compared to 44% of superficial tumors (Ta-T1). Therefore, inactivation of *RASSF1A* correlated with advanced tumor stage. Maruyuma et al. (2001) detected *RASSF1A* methylation in 35% of bladder cancers and inactivation was significantly correlated with several parameters of poor prognosis (grade 3, non-papillary, and muscle invasion) and high risk.

Inactivation of *RASSF1A* in primary prostate cancer was investigated by three groups (Table 2). Maruyama et al. (2002) detected aberrant *RASSF1A* methylation in 53% of carcinoma and *RASSF1A* inactivation was significantly associated with high preoperative serum prostate-specific antigen (P=0.005) and a high Gleason score (P<0.0001). Kuzmin et al. (2002) found complete methylation of the *RASSF1A* promoter in seven (63%) out of 11 micro-dissected prostate carcinomas and the

remaining four samples (37%) were partially methylated. Inactivation of *RASSF1A* was found in five prostate cancer cell lines, although no silencing of *RASSF1A* was found in four other prostate cancer cell lines, which were adapted for cell culture after transformation with human papillomaviral DNA. This could reflect a mutually exclusive correlation between *RASSF1A* inactivation and human papilloma virus infection, which may both play important roles in neoplastic transformation and immortalization of prostate epithelial cells (Kuzmin et al., 2002). Liu et al. (2002) found *RASSF1A* inactivation in 71% of primary prostate tumors and the methylation frequency was higher in more aggressive tumors, compared with less malignant tumors. For instance, tumors with a Gleason score of 7-10 were significantly more methylated compared with Gleason 4-6 tumors (P=0.032).

LOH at 3p21 is less common in pancreatic and colorectal carcinoma (CRC). Three different groups investigated the aberrant methylation of *RASSF1A* in colon cancer (Table 2). Yoon et al. (2001) detected *RASSF1* hypermethylation in 12% of CRC and another group observed *RASSF1A* inactivation in 45% of tumors (Wagner et al., 2002). Van Engeland et al. (2002) found *RASSF1A* promoter hypermethylation in 20% of CRC. Additionally, K-Ras mutations (codon 12 and 13) were detected in 39% of tumors. Interestingly, inactivation of *RASSF1A* occurs predominantly in CRC without alteration of K-Ras itself (P=0.023), and may provide an alternative pathway for affecting Ras signaling (van Engeland et al., 2002). Our own results suggest a similar inverse correlation between K-Ras mutations and *RASSF1A* methylation in pancreatic carcinoma (Dammann et al.,unpublished).

The methylation status of the *RASSF1A* promoter was analyzed in 38 primary thyroid tumors (Schagdarsurengin et al., 2002). In 71% of thyroid carcinoma (TC) the *RASSF1A* CpG island was hypermethylated and in thyroid cancer cell lines the *RASSF1A* promoter was completely methylated and expression was absent. Treatment of these cell lines with the DNA methylation inhibitor 5-aza-CdR reactivated the transcription of RASSF1A. Methylation frequency was higher in the aggressive forms of thyroid carcinoma, and was found in 80% of medullary TC, in 78% of undifferentiated TC and in 70% of follicular TC, compared to 62% in the more benign papillary TC (Schagdarsurengin et al., 2002). *RASSF1A* inactivation was detected in all stages of thyroid carcinoma.

Hypermethylation of the *RASSF1A* promoter was also investigated in primary pediatric tumors (Table 2). In Wilms tumor, a common childhood kidney tumor *RASSF1A* inactivation was frequently detected in 42% to 73% of specimens (Ehrlich et al., 2002; Harada et al., 2002; Wagner et al., 2002). No association was detected between CpG island hypermethylation and global DNA hypomethylation (Ehrlich et al., 2002). Harada et al. (2002) observed frequent methylation of *RASSF1A* in 88% of medulloblastoma, in 61% of rhabdomyosarcoma,

in 59% of retinoblastoma, in 52% of neuroblastoma, in 19% of hepatoblastoma, and in 15% of acute leukemia. However, *RASSF1A* inactivation was not found in osteosarcoma, in Ewing's sarcoma, and in non-malignant tissue.

In summary, *RASSF1A* hypermethylation is one of the most frequent alterations in primary human cancers and was detected at a much higher frequency compared to several other tumor suppressor genes in carcinomas. Thus, *RASSF1A* inactivation may contribute to the pathogenesis of many different forms of cancer. In malignant mesothelioma and gastric cancer, *RASSF1A* methylation was correlated with virus infection of SV40 and EBV, respectively (Toyooka et al., 2001; Kang et al., 2002). However, an inverse association was observed between viral infection and *RASSF1A* methylation in HPV-transformed prostate cancer cell lines (Kuzmin et al., 2002) and in primary cervical cancer samples (Liu et al. unpublished). Furthermore, a significant correlation was reported between *RASSF1A* methylation and impaired lung cancer patient survival (Burbee et al., 2001; Tomizawa et al., 2002) and *RASSF1A* silencing was correlated with several parameters of poor prognosis and advanced tumor stage. For several cancers, the frequency of *RASSF1A* methylation corresponds with the LOH frequency at 3p21.3. This suggests that at least for SCLC tumorigenesis *RASSF1A* inactivation of both alleles is a critical event (Agathanggelou et al., 2001; Burbee et al., 2001). In other types of cancer *RASSF1A* promoter methylation together with LOH are less frequent in the same samples (Agathanggelou et al., 2001; Burbee et al., 2001). *RASSF1A* may belong to the class of haplo-insufficient tumor suppressor genes that promote tumor formation through the inactivation of only one allele and a combination of inactivation of other 3p tumor suppressor genes (*VHL*, *FHIT* and *DUTT1/ROBO1*) might be responsible for tissue-specific carcinogenesis (Huebner, 2001). A second possibility is that another genetic lesion inactivates the pathway in which *RASSF1A* inhibits tumorigenesis.

Involvement of *RASSF1A* in Ras-associated transformation

Activated Ras is usually associated with enhanced proliferation, transformation and cell survival (Fig. 3). However, Ras also induces growth inhibitory effects (Bar-Sagi and Feramisco, 1985; Serrano et al., 1997) and apoptosis (Mayo et al., 1997; Chen et al., 1998; Downward, 1998; Shao et al., 2000). Ras effectors, like RASSF1A, may be specialized to inhibit proliferation and to induce apoptosis and these inhibitory signaling pathways may need to be inactivated during carcinogenesis (Fig. 3). Shivakumar et al. (2002) have observed that *RASSF1A* can induce cell-cycle arrest by engaging the Rb family cell-cycle checkpoint, since E7 papilloma virus protein-expressing cells are resistant to the RASSF1A-induced cell-cycle arrest. RASSF1A also inhibits accumulation of native cyclin D1 (Fig. 3) and

the RASSF1A-induced growth arrest can be relieved by ectopic expression of cyclins, but not by oncogenic Ras expression (Shivakumar et al., 2002). Vos et al. (2000) have shown that RASSF*1* binds RAS in a GTP-dependent manner and over-expression of *RASSF1* induced apoptosis. Interestingly, this pro-apoptotic effect of transient *RASSF1* transfection is enhanced by activated Ras and inhibited by dominant negative Ras. However, they used the C form of *RASSF1* in their experiment, which contains an RA domain but is not epigenetically inactivated in tumors. Other data indicate that binding of RASSF1A may require the heterodimerization with NORE1, and that RASSF1A binds to Ras only weakly by itself (Ortiz-Vega et al., 2002). Khokhlatchev et al. (2002) showed that RASSF1A and NORE1 interact with the pro-apoptotic kinase MST1, which mediates the apoptotic effect of Ras.

In a normal cell, equilibrium exists between the growth-activating function of Ras and inhibiting signals of *RASSF1A* (Fig. 3). Ultimately, any changes in this equilibrium would lead to cancer. Loss of *RASSF1* expression by epigenetic inactivation may shift the balance of Ras activities towards a growth-promoting effect without the necessity of Ras-activating mutations and vice versa. For instance, in colorectal cancer, *RASSF1A* inactivation occurs predominantly in tumors without alteration of K-ras itself, and may provide an alternative pathway for affecting Ras signaling (van Engeland et al., 2002). Our own unpublished results suggest a similar inverse correlation between K-ras mutation and *RASSF1A* methylation in pancreatic carcinoma. Additionally, Ras mutations are found in less than 1% of SCLC (Mitsudomi et al., 1991; Wagner et al., 1993), whereas inactivation of *RASSF1A* was observed in 80% and 100% of tumors by hypermethylation and LOH, respectively. The observation that *RASSF1A* inactivation and K-ras activation are mutually exclusive events in the development of certain carcinomas could further pinpoint the function of *RASSF1A* as a negative effector of Ras.
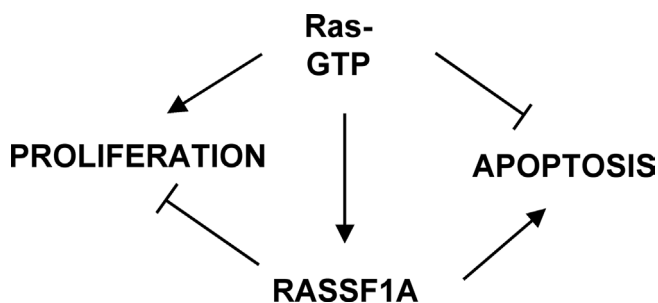

Fig. 3. Model of Ras- and RASSF1A-induced tumorigenesis. In normal cells an equilibrium exists between Ras-signaling transduction and *RASSF1A* function. Oncogenic activation of Ras or epigenetic inactivation of *RASSF1A* induces cell proliferation and inhibits apoptosis.

Tumor suppressor function of RASSF1A

Epigenetic inactivation per se is not sufficient to prove the involvement of a bona fide tumor suppressor gene in carcinogenesis (Baylin and Herman, 2001). However, since lung cancer-prone family studies are rare in the literature, genetic factors may not contribute greatly to the development of lung tumors. Therefore, inactivating germline mutations of *RASSF1A* are rather unexpected. However rare polymorphism of *RASSF1A* could show a predisposition for lung cancer development. Another proof for a tumor suppressor function is reinsertion of *RASSF1A* into cancer cell lines, which lack endogenous transcription. In lung cancer cell lines, kidney cell lines and prostate cancer cell lines reinsertion of *RASSF1A* led to reduced colony formation and/or anchorage-independent growth in soft agar (Dammann et al., 2000; Burbee et al., 2001; Dreijerink et al., 2001; Kuzmin et al., 2002). Mutant *RASSF1A* (Cys65Arg and Val211Ala) had only reduced growth suppression compared to a wild-type construct (Dreijerink et al., 2001). Tumor formation of human cancer cells was also analyzed in nude mice. Cells lacking *RASSF1A* transcription formed bigger tumors compared to the same cells expressing exogenous *RASSF1A* (Dammann et al., 2000; Burbee et al., 2001). However, ectopic expression of the RASSF1C isoform showed only a modest reduction of cell viability in vitro (Ji et al., 2002). Thus, reinsertion of *RASSF1A* inhibits tumorigenicity in vitro and in vivo.

Mouse models of human cancer may advance our understanding of carcinogenesis, and knockouts of *RASSF1A* may reveal its function. Transgenic mice expressing the SV40 large T antigen, show a high frequency of LOH in pancreatic insulinomas and intestinal carcinoid tumors on a locus on chromosome 9, named Loh-1, which lies in a region with synteny conservation to human chromosome 3p21 (Dietrich et al., 1994; Smith et al., 2002). Therefore, it will be interesting to investigate if mice with heterozygous or homozygous deletions of *RASSF1A* are tumor-prone. Smith et al. (2002) created a mouse with a 370 kb deletion of the region homologue to the 3p21.3, which includes Rassf1a. The homozygous deletion of this region is embryonic lethal in mouse. However, heterozygote mice developed normally despite being haplo-insufficient for 12 genes including *Rassf1* (Smith et al., 2002).

The *RASSF1A* gene has at least four characteristic features, which could be linked to its tumor suppressor function: 1) the Ras-association domain, which interacts with Ras in a pro-apoptotic pathway (Vos et al., 2000; Khokhlatchev et al., 2002; Ortiz-Vega et al., 2002); 2) the protein kinase C1 domain, which may bind the lipid second messenger, diacylglycerol, and the phorbol ester tumor promoter and which suggests an involvement in carcinogenesis (Kazanietz, 2000); 3) the putative ATM kinase phosphorylation consensus site, which may link *RASSF1A* to the DNA-damage response pathway (Kim

et al., 1999); and 4) the ability of *RASSF1A* to reduce cell growth and to inhibit cyclin D1 accumulation (Dammann et al., 2000; Shivakumar et al., 2002). However, the importance of these potential tumor-suppressing functions needs to be further investigated.

Conclusions

The high frequency of epigenetic inactivation of the *RASSF1A* gene in a variety of primary tumors and the ability of *RASSF1A* to inhibit growth of cancer cells suggests that this gene may function as a tumor suppressor at segment 3p21.3. K-Ras mutation and *RASSF1A* inactivation are mutually exclusive events in certain cancers and *RASSF1A* may function as a negative effector of Ras. *RASSF1A* inactivation was significantly higher in advanced-stage tumors and poorly-differentiated tumors, and epigenetic silencing of *RASSF1A* correlates with poor prognosis and impaired survival of the cancer patient. Methylation analysis of the *RASSF1A* gene could serve as the basis of a diagnostic marker for cancer. Understanding the molecular abnormalities and the function of *RASSF1A* in cancer may lead to the identification of future targets for gene therapy approaches and to the development of novel methods for the treatment of cancer.

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