Hypothesis/Commentary

Adiposity and Age are Statistically Related to Enhanced RASSF1A Tumor Suppressor Gene Promoter Methylation in Normal Autopsy Kidney Tissue

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Abstract

Age, adiposity, and smoking are risk factors for the development of renal cell carcinoma. Hypermethylation of the RAS association domain family 1A gene (RASSF1A) promoter belongs to the most frequently detected epigenetic alterations in human cancers including renal cell carcinoma. RASSF1A is functionally involved in cell cycle control in normal cells and depletion promotes a number of cellular changes increasing the risk for neoplastic growth. We investigated the hypothesis that age, modulated by the factors adiposity and anthracosis as a surrogate for smoking, is a predictor of RASSF1A promoter methylation in normal kidney tissue. Using a cross-sectional study design, we quantitatively analyzed RASSF1A methylation in 78 normal autopsy kidney tissues by quantitative combined bisulfite and restriction analysis and bisulfite sequencing, and statistically evaluated the degree of relative methylation for a relationship with the predictor age and study factors adiposity and state of anthracosis. Statistical analysis showed that age (regression analysis; \( P < 0.001 \)) and adiposity (univariate analysis; \( P = 0.016 \)) and state of anthracosis (t test; \( P = 0.005 \)) are each significantly associated with an increase of RASSF1A promoter methylation in normal kidney tissue. However, only age \( (P = 0.008) \) and adiposity \( (P = 0.008) \) were identified as independent predictors of RASSF1A promoter methylation using covariance analysis. This study provides statistical evidence that the common cancer risk factors age and adiposity enhance RASSF1A promoter methylation in nonmalignant kidney tissue. (Cancer Epidemiol Biomarkers Prev 2007;16(12):2526–32)

Introduction

Epidemiologic studies show that age, adiposity, and smoking are risk factors for the development of clear cell renal cell carcinoma (CC-RCC), the most common histologic subtype of sporadic kidney tumors (1-4), and represents the seventh most frequent cause of tumor-dependent death among men (5, 6).

Tumorigenesis of CC-RCC is frequently associated with loss and/or alteration of the short arm of chromosome 3 (7). Functional inactivation of the von Hippel-Lindau gene (VHL), a tumor suppressor gene located on 3p25 (8), has been found largely due to chromosomal alterations and point mutations, whereas promoter methylation is infrequently observed (9-11). Recently, it has been reported that the RAS association domain family 1A gene (RASSF1A), located on 3p21.3 in chromosomal proximity to VHL, is also involved in the development of RCC. Epigenetic silencing of the RASSF1A tumor suppressor gene has been detected to occur frequently in CC-RCC (12-15). Promoter hypermethylation in RCC has also been observed for the NORE1A, TIMP3, DAPK, and MT1G genes (16, 17), however, methylation frequencies seem to be lower when compared with RASSF1A, and epigenetic silencing in CC-RCC or gene function has not been analyzed in detail thus far.

RASSF1A, on the other hand, has been found to be involved in cell cycle control (18, 19). Interactions with the proapoptotic kinase MTS1 and apoptosis-inducing IFN pathways have been described (20, 21). Moreover, depletion of RASSF1A is associated with accelerated mitotic progression (19), increased risk for chromosomal defects (22), mitotic abnormalities (23), enhanced cellular motility (24), and increased tumor susceptibility in knockout mice (25).

RASSF1A promoter methylation analyses have altogether given inconsistent results when tumor-associated methylation was compared with methylation as observed in adjacent normal (peritumoral) tissue. Both considerable methylation (12, 15, 26, 27) and the absence of (or rare) peritumoral methylation (13, 14, 16) have been described, whereas two studies found no difference for the frequency of tumoral and peritumoral methylation (12, 15). On the other hand, none of these studies
specifically aimed at the comparison between tumor and peritumoral RASSF1A promoter methylation. Therefore, either a small sample size was investigated (15) or methylation results obtained by combined bisulfite and restriction analysis (COBRA) from tumor tissues were compared with methylation data gained from peritumoral tissue using methyl-specific PCR (12). Recently, we have shown that methylation in tumor tissues is significantly increased when compared with matched peritumoral tissue samples (27). Interestingly, methyl-specific PCR analysis of a panel of genes has provided suggestive evidence that hypermethylated RASSF1A promoters can also be detected in normal autopsy kidney tissue and are found with higher frequency in tissue samples of elder donors (28). Promoter methylation, characterized by a detectable onset within the second decade of life and low rate accumulation with age, has been previously described for the estrogen receptor gene in normal colonic mucosa (29).

This process has been designated as “type A” methylation (30), depending largely on the replication activity of cells in normal tissue, which might be additionally accelerated by external factors such as inflammation or carcinogens (31). It has been hypothesized that type A methylation might be an early event in tumorigenesis preceding the clonal expansion of mutated cells (32-34).

Considering that epigenetic silencing of this gene has frequently been observed in CC-RCC, RASSF1A promoter methylation, as observed in normal kidney tissue, might be the result of an accumulation process occurring in aging tissues and increasing the risk for development of RCC.

Thus, a demonstration of the relationship of age and RASSF1A promoter methylation in normal kidney tissue would provide a possible molecular link between the known statistical association of age and increased risk for RCC development. On the basis of this rationale, we did a cross-sectional study to test the hypothesis that age is a predictor of methylation of RASSF1A promoter in normal tissue.

Taking into account that epidemiologic studies identified lifestyle factors, adiposity, and smoking are also associated with an increased RCC risk, and that type A promoter methylation of tumor suppressor genes may be accelerated by external factors, we further hypothesized that adiposity and anthracosis, as an indicator of smoking, affect age-dependent methylation of the RASSF1A promoter.

In the current study, we quantitatively analyzed RASSF1A methylation in 78 normal autopsy kidney tissues and statistically evaluated the degree of relative methylation with respect to the predictor age and the study factors adiposity and status of anthracosis of tissue donors.

Materials and Methods

Study Design. To assess the possible effect of RCC risk factors age, adiposity, and anthracosis on the study outcome, the degree of relative methylation of the RASSF1A tumor suppressor gene promoter in normal autopsy renal tissue samples were analyzed in a cross-sectional study.

Tissue Samples and Characteristics. Seventy-eight normal cortical kidney tissue samples were obtained during postmortem examination no later than 24 h after death, provided that no evidence for malignancy could be detected during autopsy of donors. Tissue samples were snap-frozen in liquid nitrogen and stored at −80°C. The autopsy tissue group consisted of 28 (35.9%) female and 50 (64.1%) male tissue donors (ages 0.5-90 years; mean age, 48.6 years). Tumoral specimens were obtained

| Table 1. Pathologic and clinical characterization of the tumor group |
|---------------------------------|-----------------|-----------------|-----------------|
| Patients, n                     | 56              |
| Mean age (y)                    | 61              |
| Males, n (%)                    | 41 (73.2)       |
| Females, n (%)                  | 15 (26.8)       |
| Pathologic stage, n (%)         |                 |
| T1                              | 20 (35.7)       |
| T2                              | 10 (17.9)       |
| T3                              | 23 (41.1)       |
| T4                              | 3 (5.3)         |

*See Materials and Methods for definition of BMI groups.

| Table 2. Clinical characteristics of the BMI group |
|---------------------------------|-----------------|-----------------|-----------------|
| Normal* (BMI, 20-25)           | 30              |
| Overweight* (25 < BMI < 30)     | 20              |
| Adiposity* (BMI, ≥30)          | 17              |
| n                               |                 |
| Mean BMI                        | 22.6            |
| Mean age                        | 50.1            |
| mean age                        | 27.5            |
| mean age                        | 50.4            |
| mean age                        | 35.7            |
| mean age                        | 49.9            |

Figure 1. Calibration of COBRA analysis using plasmid controls or DNA isolated from HEK293 and CaSki cell lines as biological controls. Linear fittings of plasmid controls (solid lines) and cell line controls (dashed lines).
from 56 primary CC-RCCs after nephrectomy (age of patients, 22-90 years; Table 1), snap-frozen in liquid nitrogen and stored at −80°C. The tumor tissue group included 15 (26.8%) females and 41 (73.2%) males. Comparing the normal (autopsy) and tumor tissue group, no significant difference regarding gender was detected ($P = 0.390$, $\chi^2$ test, two-sided and Yates corrected).

**Categorization of Risk Factors Body Mass Index and Anthracosis.** The body mass index (BMI) of tissue donors was calculated as weight divided by height squared (kg/m²). BMI classes were defined according to Pischon et al. (2) and van Dijk et al. (3) as normal weight (BMI, 20-25), overweight (25 < BMI < 30), and adiposity (BMI, ≥30). Mean BMI and age of each group are shown in Table 2. Anthracosis as a proxy for smoking was evaluated during autopsy and dichotomized into the absence or presence of corresponding lung alterations.

**Isolation and Conversion of DNA.** DNA isolation, bisulfite conversion of extracted DNA, and methylation analysis were carried out as described previously (27).

**Calibration of Quantitative COBRA.** To show that COBRA can be applied for the determination of the relative degree of \(RASSF1A\) promoter methylation, defined mixtures of corresponding plasmid DNA as well as of DNA isolated from HEK293 and CaSki cell lines, which were applied as methylation-positive and -negative controls, were measured as described previously (27). The relative degree of methylation obtained by COBRA was compared with the known input ratio of methylated and unmethylated sequences. Linearity was obtained for almost two orders of magnitude both for the plasmid and cell line controls (Fig. 1). Using the 3-fold standard deviation of signals obtained from multiple negative controls, an analytic sensitivity of 2.75% of relative methylation was calculated for COBRA (data not shown).

**Bisulfite Sequencing.** For pool sequence analysis, a mixture of individual and normalized amplicons were prepared for each tissue group, cloned into pGEM-T Easy vectors (Promega) and sequenced using SP6 primer, BigDye Terminator v1.1 Cycle Sequencing Kit on an ABI 3100 Avant automated sequencer (Applied Biosystems) according to the manufacturer’s protocols or by custom sequence analysis (MWG Biotech).

**Figure 2.** A, age-related methylation in normal renal parenchyma. Relative methylation in 78 normal kidney samples was measured using COBRA and was plotted against age. Regression analysis showed a significant and robust statistical relationship between relative degree of methylation and age of tissue donors ($P < 0.001$). Curves, 95% confidence intervals of regression line. B, comparison of relative methylation of \(RASSF1A\) promoter as detected in normal weight (BMI, 20-25), overweight (25 < BMI < 30), and adiposity group (BMI, ≥30). No significant differences were observed for the covariate age in the three BMI groups (see Table 2). C, age-related methylation in the normal weight (Δ) and adiposity (■) group. Linear fittings of the corresponding relative methylation values from the normal weight group (solid lines) and from the adiposity group (dashed lines).
Statistical Analysis. Statistical analyses were done using the SPSS statistical software (Statistical Package for Social Sciences, SPSS, Inc.). \( P \leq 0.05 \) was considered statistically significant. A possible statistical association of our study predictor, variable age, and the study outcome, the relative degree of RASSF1A promoter methylation in tissues, was assessed using a regression analysis. To analyze a possible effect of the factor adiposity on relative methylation, we used BMI as a categorical variable including the categories normal, overweight, and adiposity as specified in Table 2. Sixty-seven of 78 (86%) autopsy samples could be considered for further statistical analysis; 11 donors did not fulfill the inclusion criteria requiring a normal, overweight, or adiposity category of BMI.

The relationship of normal, overweight, or adiposity categories and the study outcome was first analyzed using univariate analysis. Covariance analysis was applied to analyze the association of the covariate age and the cofactors adiposity and anthracosis. Pairwise comparisons of the normal, overweight, or adiposity groups in covariance analysis were carried out considering the adjustment for multiple comparisons according to Bonferroni. The statistical relationship of the dichotomous variable anthracosis and the study outcome was analyzed using a \( t \) test. For comparison of relative methylation as observed in the normal, tumor, and negative control group, one-way ANOVA was carried out. Sequencing data obtained from tumor and normal tissue sequence pools were statistically compared using the number of methylated CpG sites and the \( \chi^2 \) test.

Results

The relative degree of RASSF1A promoter methylation was determined in 78 normal kidney samples with the use of quantitative COBRA and plotted against the age of the tissue donors (Fig. 2A). It can be seen that individual samples showed a detectable onset of methylation during first two decades of life of the tissue donors. Regression analysis showed a highly significant and robust relationship between the relative degree of methylation observed in tissue samples and age \( (P \leq 0.001) \). The slope of the regression line indicated an average increase of methylation of 0.16% per year. Our regression analysis further showed that a considerable statistical spread of methylation values from tissues of a comparable age was observed. A Pearson's coefficient of correlation of 0.51 was determined, indicating that other factors besides age might affect the relative methylation values measured in normal kidney tissue.

To identify a potential statistical association between the BMI categories normal, overweight, and adiposity and the study outcome, relative degree of RASSF1A promoter methylation, univariate analysis was first carried out (Table 3). This analysis showed that the BMI categories represent a significant factor for RASSF1A promoter methylation in normal tissue \( (P = 0.018) \). Pairwise comparison revealed a significant difference between the normal and adiposity BMI categories \( (P = 0.016; \text{Bonferroni adjusted}) \).

The effect of the dichotomous variable anthracosis on tissue methylation levels was analyzed using the \( t \) test. We found significantly increased methylation in the anthracosis-positive group (mean methylation, 10.3%) when compared with tissue from anthracosis-negative donors (mean methylation, 7.3%; \( P = 0.005 \)).

Covariance analysis was carried out to assess the effects of the categorical variable BMI, the dichotomous variable anthracosis, and covariate age on the study outcome in a combined statistical model. Analysis of covariance revealed that age represents a significant covariate \( (P = 0.008) \) and BMI classification is a significant factor \( (P = 0.008) \), whereas no significance was obtained for the factor anthracosis \( (P = 0.186) \). Pairwise comparisons based on Bonferroni adjustments identified a significant difference in methylation of RASSF1A between the normal weight group (BMI, 20-25; mean methylation, 7.5%) and the adiposity group (BMI, ≥30; mean methylation, 11.5%; \( P = 0.007) \).

After the identification of adiposity as an independent factor for RASSF1A promoter methylation in normal kidney, we graphically compared age-dependent methylation in the normal weight and the adiposity group (Fig. 2C). Linear fitting suggests that the accumulation of methylation due to age could be accelerated in the adiposity group.

To evaluate COBRA measurements of individual autopsy tissue samples, we compared these values to the relative degree of RASSF1A methylation obtained from 56 individual tumor samples as positive controls, and plasmid-encoded DNA as negative controls (Fig. 3A). One-way variance analysis and pairwise comparisons according to the Bonferroni adjustment showed significant differences between the degree of relative methylation of the autopsy (mean relative methylation, 9.1%), the tumoral (mean relative methylation, 20.7%), and the control groups (mean relative methylation, 1.8%; ANOVA, \( P < 0.0005 \); normal versus positive control, \( P < 0.0005 \); normal versus negative control, \( P = 0.022 \)).

In addition to the measurements of individual kidney tissues, as carried out by quantitative COBRA, we conducted a comparison in groups of pooled sequences each obtained from the autopsy tissue group and the positive and negative control groups using bisulfite sequencing analysis (Fig. 3B). Thirty-eight (6.1%) of 624 CpG sites were found to be methylated in the normal tissue group, whereas 67 (10.5%) of 640 sites.

### Table 3. Statistical relationship of RASSF1A promoter methylation and RCC risk factors age, adiposity, and anthracosis

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<th>Statistical analysis</th>
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<td>Age</td>
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<td>Normal-adiposity</td>
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<td>Anthracosis</td>
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<td>Anthracosis</td>
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*Bonferroni adjusted.
and 3 (1%) of 320 sites were found to be methylated in the positive and negative control groups, respectively ($P < 0.0005$, $\chi^2$ test).

**Discussion**

The study shows that promoter methylation of *RASSF1A* in normal kidney tissue as the primary study outcome variable is statistically associated with the study predictor variable age ($P \leq 0.001$), thus supporting the study hypothesis that age is a predictor of *RASSF1A* promoter methylation in normal kidney. Methylation of *RASSF1A* is detectable within the first two decades of life and accumulates at a low rate of $\sim 0.2\%$ per year in the aging normal kidney tissue. Both the commencement of detectable promoter methylation and the rate of accumulation are similar to the methylation characteristics described earlier for the estrogen receptor gene in normal colon tissue samples (29).

This study also shows that adiposity increases *RASSF1A* promoter methylation in normal kidney samples when compared with the normal weight donor group. This effect is statistically independent from the covariate age and the cofactor anthracosis. Hence, our analysis supports the hypothesis that adiposity is a cofactor of *RASSF1A* promoter methylation in normal kidney. On the other hand, our analysis of relative methylation shows variations within normal tissues of comparable age; from a statistical point of view, this cannot be explained by the cofactor adiposity alone. Comparison of tissue donors exhibiting anthracosis—which to some extent is assumed to reflect nicotine abuse—versus tissue contributors without detectable alterations in the lung showed significantly increased methylation of *RASSF1A* in univariate analysis ($P = 0.005$) but failed to exhibit a significant difference in covariance analysis ($P = 0.186$). However, when considering that the increase in mean methylation due to anthracosis is $\sim 30\%$ lower when compared with mean relative methylation noted for donors of the adiposity group, it is likely that a specifically adjusted study design is required to clarify whether anthracosis/smoking is an independent factor of age-related and adiposity-affected *RASSF1A* promoter methylation.

Thus, the study was limited by the sample size, which did not permit significant statistical differentiation of the small difference observed between the increase of

**Figure 3.** A, in groups, comparison of relative methylation of *RASSF1A* promoter as measured by COBRA in 78 normal (N) and 56 tumor (T) kidney tissue samples, and negative, plasmid-encoded DNA controls (C). Normal tissue samples obtained from autopsy tissue were statistically different from signals obtained from negative controls. Furthermore, a significant difference of methylation was found within tumor and normal tissue samples. B, bisulfite sequencing of tumoral and normal kidney *RASSF1A* promoters. *RASSF1A* promoter amplicons from both groups were pooled and cloned. Thirty-nine and 40 clones of the normal kidney (N) and tumor (T) tissue group, respectively, were analyzed by sequencing. The methylation status of analyzed CpG sites for each clone (●, methylation; ○, no methylation).
methylation due to either adiposity or anthracosis. Furthermore, only RASSF1A tumor suppressor gene promoter methylation was considered in this study although other genes might also be affected by adiposity.

However, hypermethylation of the RASSF1A promoter is one of the most frequent epigenetic alterations detected in tumors, and the protein functionally contributes to mitosis, chromosomal stability, and apoptosis. Considering that RASSF1A promoter methylation is likely involved in the early tumorigenesis of CC-RCC (12, 27), we assessed RASSF1A as the currently most interesting target for age- and lifestyle factor–related promoter methylation analyses in normal kidney tissue.

This study shows that a statistically significant relationship exists connecting age and adiposity to the extent of RASSF1A promoter methylation in normal kidney tissue. However, whether partial promoter methylation, as observed in large part from normal kidney tissues, diminishes the expression of RASSF1A has not yet been analyzed. On the other hand, in view of the seed and spread theory described recently (31), partial methylation in normal tissue is assumed to represent an intermediate state of methylation that might finally lead to promoter hypermethylation associated with gene silencing and heterogeneous expression in normal tissue. Recently, we detected a mosaic-like distribution of RASSF1A expression in histopathologically normal kidney tissue (27), supporting the assumption that hypermethylation and epigenetic silencing of RASSF1A may occur in a subset of normal kidney tissue cells.

Epidemiologic evidence has been reported that BMI represents a risk factor for colon, prostate, gallbladder, endometrial, and breast cancer (35, 36), as well as RCC (3, 37). Accordingly, it has been hypothesized that a lack of physical activity is associated with increased methylation of tumor suppressor genes in normal breast tissue (38). Some recent findings could provide a biological explanation for the statistical relationship between BMI and increased promoter methylation in normal kidney tissue. It has been described that obesity is associated with elevated serum levels of insulin-like growth factor I, which is capable of promoting cellular proliferation and inhibiting apoptosis (39). Thus, the mitogenic effects of insulin-like growth factors have been shown in various cancers, and kidney proximal tubule cells as well as renal carcinoma cells are responsive to insulin-like growth factor-I (40, 41). Furthermore, it is known that adipose tissue in visceral obesity releases adipokines, which are associated with an increase of proinflammatory cytokines such as tumor necrosis factor-α and interleukin-6 (42, 43). Thus, both insulin-like growth factors and proinflammatory cytokines might contribute to increased methylation considering that methylation spreading in normal tissue has been described to be driven by replication (31), which in turn, is enhanced by mitogenic factors as well as inflammation.

Conclusions
To our knowledge, promoter methylation of a tumor suppressor gene in general, and in particular of RASSF1A, has not yet been described to be associated with adiposity in normal tissue. Conclusively, this study provides statistical evidence that RASSF1A promoter methylation possibly represents a molecular link between RCC tumorigenesis and the RCC risk factors age and adiposity. Our study shows that anthracosis/smoking could also be a modulator of RASSF1A promoter methylation in normal kidney, although further analysis is required to statistically differentiate between adiposity- and anthracosis-related effects following methylation.

Moreover, this study raises the question of whether other normal tissues might also show age- and adiposity-dependent RASSF1A promoter methylation. Considering that age-dependent methylation has already been detected in several gene promoters, we suppose that other combinations of gene promoters and tissues might also be prospectively identified demonstrating adiposity-affected and age-dependent methylation. Furthermore, it would be of great interest to analyze the biological mechanism of how methylation is enhanced by adiposity and whether changes in lifestyle might reverse promoter methylation levels of the RASSF1A tumor suppressor gene in normal kidney tissue.

References


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