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Cancer-testis gene expression is associated with the methylenetetrahydrofolate reductase 677 C>T polymorphism in non-small cell lung carcinoma

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Abstract

Background: Tumor-specific, coordinate expression of cancer-testis (CT) genes, mapping to the X chromosome, is observed in more than 60% of non-small cell lung cancer (NSCLC) patients. Although CT gene expression has been unequivocally related to DNA demethylation of promoter regions, the underlying mechanism leading to loss of promoter methylation remains elusive. Polymorphisms of enzymes within the 1-carbon pathway have been shown to affect S-adenosyl methionine (SAM) production, which is the sole methyl donor in the cell. Allelic variants of several enzymes within this pathway have been associated with altered SAM levels either directly, or indirectly as reflected by altered levels of SAH and Homocysteine levels, and altered levels of DNA methylation. We, therefore, asked whether the five most commonly occurring polymorphisms in four of the enzymes in the 1-carbon pathway associated with CT gene expression status in patients with NSCLC.

Methods: Fifty patients among a cohort of 763 with NSCLC were selected based on CT gene expression status and typed for five polymorphisms in four genes known to affect SAM generation by allele specific q-PCR and RFLP.

Results: We identified a significant association between CT gene expression and the MTHFR 677 CC genotype, as well as the C allele of the SNP, in this cohort of patients. Multivariate analysis revealed that the genotype and allele strongly associate with CT gene expression, independent of potential confounders.

Conclusions: Although CT gene expression is associated with DNA demethylation, in NSCLC, our data suggests this is unlikely to be the result of decreased MTHFR function.

Keywords: Cancer-testis genes, 1-carbon pathway, Adomet, DNA methylation

Background

Cancer-testis (CT), or cancer-germline genes, currently with more than 100 members, are distinctly expressed in cancer, germline and trophoblast cells but not in other normal tissues in the adult. Most CT genes constitute multigene families organized in clusters along the X chromosome. Members within a family are highly homologous, however, no conservation of sequence exists between families [1]. Despite the lack of sequence similarity (including promoters), re-expression of almost all CT genes in tumors correlates with the demethylation of their promoters that occurs in parallel to a genome-wide demethylation event, primarily affecting repeat regions [2]. The mechanisms leading to CT gene promoter demethylation in cancer are unknown. Increased BORIS expression has been associated with upregulated CT gene expression [3,4], but the protein is likely not the sole responsible factor in this event. Histone acetylation has also been shown to facilitate CT gene expression, primarily when it associates with DNA demethylation [5].

As most CT gene products are highly antigenic they have been utilized in clinical trials based on immunotherapeutic approaches targeting these antigens [6]. Since patient eligibility for CT targeting immunotherapy requires that the tumor express CT genes, it is important to know whether CT gene expression can be induced. It is expected...
that any approach leading to CT gene expression should also result in the demethylation of their promoters.

Production of the sole methyl donor in the cell, S-adenosylmethionine (SAM), depends on the efficient utilization of folate, by the 1-carbon pathway. Several enzymes in this pathway contain common polymorphic variants that reduce the efficiency of the enzyme and thus, the rate of SAM production. Hypomorphic alleles of four of these enzymes (methyleneetetrahydrofolate reductase (MTHFR), methionine synthase reductase (MTRR), methionine synthase (MTR), and reduced folate carrier (RFC)), have been associated with cellular under-utilization of folate and homocysteine, increased DNA hypomethylation, and decreased CpG methylation [7-11]. More recently, the hypomorphic 677 T allele of MTHFR, has been associated with the expression of MAGE-A1, a CT gene, in glioblastoma multiforme [12]. Others, however, could not reproduce these findings in ovarian carcinoma [13]. In the present study we asked if polymorphisms of the 1-carbon pathway enzymes associate with CT gene expression in non-small cell lung cancer (NSCLC) patients. Our results show a strong association between the MTHFR 677 CC genotype as well as the MTHFR 677 C allele and CT gene expression independent of age, sex, histology, and tumor stage.

Methods

Patients and tumor material

Tumor samples obtained from patients undergoing curative surgical resection for primary NSCLC at the Department of Cardio-Thoracic Surgery, Weill Medical College of Cornell University, from 1991 to July 2005 were analyzed in this study. Informed consent was obtained from all patients. The study was approved by the Institutional Review Board of Weill Medical College of Cornell University. Fifty tumor samples were selected solely based on CT gene expression from 763 samples that had been evaluated for the presence of transcripts from up to 9 CT genes (NY-ESO-1, LAGE-1, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, CT-7, SSX2, and SSX4), by semi-quantitative PCR, as described previously [14]. Twenty one samples with CT expression in at least 4 of the 9 CT genes tested, with strong expression in at least one gene, constituted the CT (+) group. Twenty-nine samples with no CT expression in any of the CT genes tested (with a minimum of 5 CT genes tested) were selected as CT (−) tumors for this study. CT gene expression was determined as strong (+++), intermediate (++), weak (+ or +/-), or none (−) as previously described [14], and is shown in Additional file 1: Table S1.

DNA analysis

Genomic DNA extracted from tumor tissues were genotyped using pre-designed 5'-nuclease TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA) using a Stratagene Mx3005P instrument according to the manufacturer’s instructions. The SNPs typed and their reference IDs were: MTHFR 677 C > T (rs1801133), MTHFR 1298 A>C (rs1801131), MTR 2756 A>G (rs1805087), and MTRR 66 A>G (rs1801394). Nested PCR-RFLP was used to type the RFC 80 G>A (rs1051266) polymorphism for which the first round PCR conditions were previously described [10]. Nested PCR primers were: 5'-AGCCGTTAGAGCAAAGGTAGC-3' and 5'-AGCCGTACCTTCGTTCC-3'. PCR was performed using DyNAzyme™ II Hot Start DNA Polymerase (Finzymes, Keilaranta, Finland). PCR conditions were: 10' activation at 94°C, followed by 35 cycles of 94°C, 62°C and 72°C; 30” each, with a final 72°C, 7’ extension. HinP1I (New England Biolabs, Hertfordshire, UK) digested PCR products were analyzed as described previously [10]. All analyses were repeated at least twice.

Genotypes for all polymorphisms were determined successfully in all cases (Additional file 2: Table S2). Genotype distributions did not deviate from Hardy-Weinberg equilibrium (Additional file 3: Table S3). Minor allele frequencies for individual loci were: 40% for MTHFR 677 C > T, 26% for MTHFR 1298 A > C, 14% for MTR 2756 A > G, 54% for MTRR 66, and 42% for RFC 80 G > A. MTHFR genotypes were not independently distributed across the 2 loci. The major 677C allele was in linkage disequilibrium with the minor 1298C allele (D’ = 0.99, r² = 0.23) [15].

In silico association analysis

Paired datasets, GSE14471 and GSE15714, containing gene expression and SNP genotyping data, respectively, from 111 pediatric acute myeloid leukemia samples (of which 109 were typed successfully), were analyzed for an association between CT gene expression and MTHFR 677 genotype distribution [16]. A principal component analysis using 44 probesets corresponding to 9 CT gene families was performed for the expression dataset. The first principal component, explaining 0.48 of variance for CT gene expression was used to generate groups representing samples with low, intermediate, and high CT gene expression by K means clustering using a customized R code [17]. Optimum number of clusters according to Elbow criterion was determined as five. Therefore, five initial cluster centers were placed equally distant from each other where the first and last centers represented the minimum and maximum values of PC1, respectively. Centers were iteratively updated based on the median value of the reassigned cluster members until no change in cluster membership took place. The five clusters were regrouped into three representing low (clusters 1 & 2), intermediate (cluster 3), and high CT gene expression (clusters 4 & 5).
Statistical analysis

To analyze the association between 1-carbon pathway enzyme polymorphisms and CT gene expression, the genotype distributions were compared in CT (+) and CT (-) tumors by Pearson’s Chi-Square (2 degrees of freedom) or Fisher’s exact tests. Odds ratios (OR) were estimated by multivariate logistic regression. To evaluate whether CT gene expression was related to sex, smoking status, tumor size, and disease stage, Fisher’s exact test or Chi-square tests were used. Race information was available for only 29 patients of which 25 were non-Hispanic white, one was a non-Hispanic black, and 3 were of mixed race, and was not included in statistical analyses. All statistical tests were two-sided with a 5% type I error rate, unless indicated otherwise, and were carried out using SAS (version 9.3) software (SAS Institute, Cary, NC). $P < 0.05$ was considered statistically significant.

Table 1 Demographics and clinical characteristics

| Demographic/Parameter | CT (+) patients (n=21) | CT (-) patients (n=29) | P*  |
|-----------------------|------------------------|------------------------|-----|
| Age                   |  >60                   | 12                     | 19  | 0.74 |
|                       | <=60                   | 6                      | 7   |     |
| Pathological tumor size | >3 cm                  | 10                     | 8   | 0.21 |
|                       | <=3 cm                 | 8                      | 17  |     |
| T stage               | 1                      | 5                      | 14  | 0.007|
|                       | 2                      | 8                      | 12  |     |
|                       | 3                      | 3                      | 0   |     |
|                       | 4                      | 2                      | 0   |     |
|                       | Unknown                | 3                      | 3   |     |
| TNM stage (Pathologic stage of primary tumor) | I                      | 9                      | 18  | 0.37 |
|                       | II                     | 5                      | 3   |     |
|                       | III                    | 4                      | 5   |     |
|                       | IV                     | 0                      | 0   |     |
|                       | Unknown                | 3                      | 3   |     |

* Chi-square (Fisher’s exact test, two sided) or chi-square test for trend; * SQCC Squamous cell carcinoma; $^\dagger$patients with missing clinical data were not included in statistical analyses.

Table 2 Distribution of individual genotypes among CT (+) and CT (-) tumors

| Polymorphism    | Genotype | CT (+) Tumors, n (%) | CT (-) Tumors, n (%) | $\chi^2$ | P* |
|-----------------|----------|----------------------|----------------------|---------|----|
| MTHFR 677 C>T   | CC       | 13 (62%)             | 7 (24%)              | 7.30    | 0.03|
| (rs1801133)     | CT       | 5 (24%)              | 15 (52%)             |         |    |
|                 | TT       | 3 (14%)              | 7 (24%)              |         |    |
| MTHFR 1298 A>C  | AA       | 13 (62%)             | 14 (48%)             | 0.91    | 0.63|
| (rs1801131)     | AC       | 7 (33%)              | 13 (45%)             |         |    |
|                 | CC       | 1 (5%)               | 2 (7%)               |         |    |
| MTR 2756 A>G    | AA       | 14 (67%)             | 22 (76%)             | 0.51    | 0.53**|
| (rs1805087)     | AG       | 7 (33%)              | 7 (24%)              |         |    |
|                 | GG       | 0 (0%)               | 0 (0%)               |         |    |
| MTRR 66 A>G     | AA       | 4 (19%)              | 9 (31%)              | 0.92    | 0.63|
| (rs1801394)     | AG       | 10 (48%)             | 12 (41%)             |         |    |
|                 | GG       | 7 (33%)              | 8 (28%)              |         |    |
| RFC 80 G>A      | GG       | 6 (29%)              | 12 (41%)             | 1.90    | 0.39|
| (rs1051266)     | GA       | 9 (43%)              | 13 (45%)             |         |    |
|                 | AA       | 6 (29%)              | 4 (14%)              |         |    |

* Chi-square (degrees of freedom); ** Fisher’s exact test, two sided.
Discussion

Among the five markers analyzed in this study, we find a strong association between the major MTHFR 677 CC genotype, as well as the MTHFR 677 C allele and CT gene expression in lung cancer. This contrasts with earlier studies where the minor allele of this SNP was associated with decreased SAM production, decreased methylation rates, and decreased MTA production, which in turn could lead to genome-wide hypomethylation and the exact SAM concentration required to find one more polymorphism significant. Therefore, analysis of larger cohorts might reveal additional associations as well as compound effects of SNPs within the 1-carbon pathway enzymes on CT gene expression. Models to test for such effects were not computed in this study due to the limited sample size.

Although decreased SAM levels might be expected to result in DNA demethylation, the exact SAM concentration threshold required for gene re-expression might be affected by various other parameters not tested in this study. A candidate is thymidylate synthase (TS) whose levels are known to fluctuate widely in cancer and which can inhibit MTHFR activity [22]. CT gene expression is associated with larger tumors and advanced stage [14]. If this is to be taken as a sign of increased proliferation, it would imply increased TS activity, and thus, possibly suppressed MTHFR, which in turn could affect CT gene expression. On the other hand, increased SAM production might indirectly inhibit methylation reactions via methylthioadenosine (MTA), a nucleoside produced from SAM through the polyamine biosynthetic pathway. MTA can induce cell cycle arrest and apoptosis, which in turn could result in DNA demethylation, the exact SAM concentration required to find one more polymorphism significant. Therefore, analysis of larger cohorts might reveal additional associations as well as compound effects of SNPs within the 1-carbon pathway enzymes on CT gene expression. Models to test for such effects were not computed in this study due to the limited sample size.

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CT gene expression. However, due to the limited power of this study, we might have missed individual or cumulative effects of SNPs within other enzymes of the 1-carbon pathway on CT gene expression. SAM/SAH ratios for the tissues analyzed here were also unknown. Hence, we only contributed to, but did not resolve this interesting story, and hope future studies reveal the intricacies of the relation between CT gene expression and genetic variants of the 1-carbon pathway enzyme genes.

Additional files

Additional file 1: Table S1. CT Gene Expression and Distribution of Clinical Parameters within NSCLC Patients.

Additional file 2: Table S2. Genotypes of NSCLC Patients.

Additional file 3: Table S3. Hardy-Weinberg Distributions of Single Nucleotide Polymorphisms in NSCLC Patients.

Additional file 4: Figure S1. Principal component analysis based in silico clustering of AML. Tumor samples are shown ordered from the lowest to the highest first principal component (PC1) value. The 5 clusters generated by K-means clustering are indicated. Tumors with low, intermediate and high CT gene expression correspond to clusters 1-2, 3, and 4-5, respectively.

Competing interests

The authors declare that they have no competing interest.

Authors’ contributions

Overall study design: KMS, MG, ZK, YTC, AOG; patient recruitment and sample collection: NKA and YTC; genotyping experiments: KMS and ARB; principal component analysis and other in silico analyses: MI, OK and KMS; statistical analyses: MG, ZK, OK and KMS. All authors read and approved the final manuscript.

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