# Association of *T-786C* eNOS gene polymorphism with increased susceptibility to acute chest syndrome in females with sickle cell disease

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## Summary

Acute chest syndrome (ACS) is a life-threatening complication of sickle cell disease (SCD). A retrospective study was performed to evaluate the role of endothelial nitric oxide synthase (eNOS) gene polymorphisms (*E298D* and *T-786C*) in African–American SCD patients. The *D298* allele showed no association; the *C-786* allele showed a statistically significant association ($P = 0{\cdot}0061$) in female ACS cases. Multiple logistic regression analysis showed that relative risk of ACS was 8·695 ($P = 0{\cdot}0076$, 95% confidence interval 1·761–42·920) for female carriers of *C-786*. eNOS *T-786C* is a gender-specific genetic modifier that is associated with increased susceptibility to ACS in female SCD patients.

**Keywords:** sickle cell disease, acute chest syndrome, nitric oxide, genetic modifier, polymorphism.

Acute chest syndrome (ACS) occurs in 15–43% of patients with SCD and is the leading cause of mortality (4%) (Platt *et al*, 1994). Recurrent episodes develop in 20–80% of patients, often progressing to pulmonary hypertension. Factors that have been associated with increased risk of ACS are haemoglobin level, fetal haemoglobin level and leucocyte count (Castro *et al*, 1994).

Nitric oxide (NO) has shown promise as a therapeutic agent for ACS. It is synthesized from L-arginine by nitric oxide synthase (NOS), which has three isoforms: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). eNOS is regulated by oestrogen and is altered by drugs, including cigarette smoke, and by many disease states, including hypercholesterolaemia, diabetes and hypertension (Li *et al*, 2002).

The identification of genetic modifiers of ACS would aid the determination of potential therapeutic targets and enable individualized treatment strategies. Two functional single nucleotide polymorphisms (SNPs) in the eNOS gene (*E298D* and *T-786C*) are associated with coronary vasospasm (Yoshimura *et al*, 1998). The *T-786C* polymorphism significantly reduces eNOS gene promoter activity (Nakayama *et al*, 1999), and the *E298D* changes an amino acid in the enzyme's oxygenase domain. The aim of this retrospective study was to examine these polymorphisms as potential modifiers for ACS and to analyse males and females separately, since gender differences in NO bioavailability have been reported (Gladwin *et al*, 2003).

## Patients and methods

### *Patients*

A total of 87 African–American patients with SCD were enrolled from two centres: Thomas Jefferson University Hospital and Nemours Children's Clinic. Patients were enrolled after informed consent during regular SCD clinic visits. Inclusion criteria were: SS or $S\beta^0$ thalassaemia and age >13 years. Clinical data regarding number and dates of ACS

episodes were obtained by retrospective chart review, with ACS identified by (i) presence of new pulmonary infiltrate(s) on chest X-ray and (ii) acute chest pain. Genomic DNA was isolated from peripheral blood using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA).

### Genotyping

The *E298D* polymorphism was genotyped by restriction fragment-length polymorphism analysis following Ban II digestion of polymerase chain reaction (PCR) products as described (Shimasaki *et al*, 1998).

The *T-786C* polymorphism was genotyped by Taqman® Allele Discrimination assay and real-time PCR on an ABI 7900 with SDS v 2.1 software [Applied Biosystems (ABI), Foster City, CA, USA]. The primer and probe sequences were eNOS-786F 5′-CCA CCA GGG CAT CAA GCT-3′ and eNOS-786R 5′-CGC AGG TCA GCA GAG AGA CTAG-3′; Probe (T) Vic 5′-CTG GC<u>T</u> GGC TGAC-3′ and Probe (C) FAM 5′-TCC CTG GC<u>C</u> GGCT-3′ (polymorphism underlined).

### DNA sequence analysis

Equivocal T-786C samples underwent DNA sequence analysis using Big Dye Terminator v 3.1 (ABI) on an ABI 3100. PCR primers were 5′-CAG CTA GTG GCC TTT CTC CA-3′ and 5′-TGC AGG TTC TCT CCT TCA CC-3′. The product was sequenced using the primer 5′-CCC TCA GAT GGC ACA GAA CT-3′.

### Statistical methods

Allele and genotype frequencies were compared by standard contingency table analysis using two-tail Fisher's exact test probabilities. Multiple logistic regression analysis utilizing SAS software (SAS Incorporated, Cary, NC, USA). Linkage disequilibrium between the SNPs was tested using the GOLD program (Abecasis & Cookson, 2000). Statistical significance was defined as a *P*-value <0·05.

## Results

Our SCD population comprised 46 non-ACS controls and 41 ACS cases (Table I). Female ACS patients were significantly older than female non-ACS patients ($P = 0{\cdot}0154$). Genotype distributions for *E298D* were *EE* 81%, *ED* 19% and *DD* 0% with the frequency of the minor allele (*D*) being 9% in the study population. Genotype distributions for *T-786C* were *TT* 77%, *TC* 20·7%, *CC* 2·3%, with the frequency of the minor allele (*C*) being 13%, similar to that found in the African–American population (Tanus-Santos *et al*, 2001). Haplotype analysis showed no significant association between the two markers ($\chi^2 = 3$, 1 degree of freedom, $P = 0{\cdot}08$; $D = 0{\cdot}242$). Therefore, in subsequent case–control analyses, the two polymorphisms were tested separately. On analysing cases and controls, no significant difference was observed in genotype or allele distributions for *E298D*. There was also no association of *T-786* with ACS in the total population; the frequency of the minor allele in all cases and all controls was 17% and 9% respectively ($P = 0{\cdot}11$). However, gender differences were evident (Table II). The frequency of the minor allele (*C*) in female cases and controls was 28% and 6% respectively ($P = 0{\cdot}0051$). Resulting genotype frequencies differed in female cases and controls (*TT* 50%, *TC* 44%, *CC* 6% vs. 89%, 11%, 0%, respectively, $P = 0{\cdot}0061$), while the genotype and allele frequencies in male cases and controls were similar. Logistic regression analysis of the effect of genotype on risk of ACS with age as a covariate showed that the differences in genotype distributions remained significant ($P = 0{\cdot}0076$) and confirmed the significant age difference between female cases and controls ($P = 0{\cdot}0260$). This analysis also confirmed that the association with the *E298D* polymorphism was not significant ($P = 0{\cdot}2666$). The odds ratio for ACS in homozygotes or heterozygotes for *T-786C* was 8·695 (95% confidence interval 1·761–42·920).

**Table I.** Patient characteristics.

| Characteristics | Non-ACS (%) | ACS (%) | Total (%) |
|---|---|---|---|
| SS | 44 (96) | 39 (95) | 83 (95) |
| S$\beta^0$ | 2 (4) | 2 (5) | 4 (5) |
| Female | 27 (59) | 18 (44) | 45 (52) |
| Male | 19 (41) | 23 (56) | 42 (48) |
| Total | 46 | 41 | 87 |
| Mean age (years) | 31 | 36 | 34 |
| Female mean age (range) | 30 (15–73) | 41 (16–71) | 34 |
| Male mean age (range) | 33 (15–69) | 32 (15–52) | 32 |

ACS, acute chest syndrome.

## Discussion

We evaluated two functional SNPs in the eNOS gene and showed that *T-786C* was associated with increased likelihood of ACS in females only. There is no known predisposition of female patients for ACS over males. Thus, our results suggest that there are different ACS genetic modifiers in males and females. The FCP locus on the X chromosome affects fetal globin production, with females having more F cells (Dover *et al*, 1992), but the gene or allele responsible for this finding has yet to be identified. eNOS *T-786C* may be the first polymorphism to be identified as a gender-specific modifier in SCD.

The data do not distinguish between *T-786C* being causative or in linkage disequilibrium with an ACS susceptibility determinant. It is unclear why males with this polymorphism do not show increased susceptibility to ACS. Interestingly, the *T-786C* site is in close proximity with a predicted oestrogen-responsive element, and a DNA binding protein, RPA1, has been shown to reduce *C-786* eNOS promoter activity (Miyamoto *et al*, 2000).

**Table II.** Genotype and allele frequency of endothelial nitric oxide synthase single nucleotide polymorphisms (SNPs) in acute chest syndrome cases and controls in patients with sickle cell disease.

| SNP | Group | Genotype count (%) | | | *P*-value (genotype)† | Allele frequency | *P*-value (allele)‡ |
|---|---|---|---|---|---|---|---|
| | | *EE* | *ED* | *DD* | | | |
| E298D | All | | | | | | |
| | Cases | 32 (78) | 9 (22) | 0 | 0·6020 | 0·11 | 0·6210 |
| | Controls | 38 (83) | 8 (17) | 0 | | 0·09 | |
| | Females | | | | | | |
| | Cases | 12 (67) | 6 (33) | 0 (0) | 0·3040 | 0·17 | 0·3367 |
| | Controls | 22 (82) | 5 (18) | 0 (0) | | 0·09 | |
| | Males | | | | | | |
| | Cases | 20 (87) | 3 (13) | 0 (0) | 1·0000 | 0·07 | 1·0000 |
| | Controls | 16 (84) | 3 (16) | 0 (0) | | 0·08 | |
| | | *TT* | *TC* | *CC* | | | |
| T-786C | All | | | | | | |
| | Cases | 28 (68) | 12 (29) | 1 (2) | 0·0793 | 0·17 | 0·1131 |
| | Controls | 39 (85) | 6 (13) | 1 (2) | | 0·09 | |
| | Females | | | | | | |
| | Cases | 9 (50) | 8 (44) | 1 (6) | 0·0061 | 0·28 | 0·0051 |
| | Controls | 24 (89) | 3 (11) | 0 (0) | | 0·06 | |
| | Males | | | | | | |
| | Cases | 19 (83) | 4 (17) | 0 (0) | 1·0000 | 0·08 | 0·7252 |
| | Controls | 15 (79) | 3 (16) | 1 (5) | | 0·13 | |

†*P*-value: comparing homozygous variant and heterozygous genotypes to homozygous wild type in cases and controls.
‡*P*-value: comparing variant allele in cases and controls.

A large, prospective study of this SNP as modifier for ACS is now needed to examine its interaction with known ACS risk factors, such as the level of anaemia and fetal haemoglobin as well as factors that influence eNOS levels (e.g., cigarette smoking, hypercholesterolaemia, hormone-replacement therapy and diabetes). It will also be interesting to determine NO bioavailability in patients of known *T-786C* genotypes.

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## Note added in proof

We also genotyped our patient population for the VNTR located in intron 4 and found that there was no association between repeat number and ACS in our total population ($P = 0{\cdot}67$), or in our female patients ($P = 0{\cdot}72$). As has been shown by Tanas-Santos *et al*, 2001 in African Americans, the VNTR was in linkage disequilibrium with the E298D SNP ($P = 0{\cdot}024$, $D' = 0{\cdot}754$).

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