DNA methylation of the long intergenic noncoding RNA 299 gene in triple-negative breast cancer: results from a prospective study

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Triple-negative breast cancer (TNBC) is an aggressive breast cancer subtype associated with a high rate of recurrence and poor prognosis. Recently we identified a hypermethylation in the long noncoding RNA 299 (LINC00299) gene in blood-derived DNA from TNBC patients compared with healthy controls implying that LINC00299 hypermethylation may serve as a circulating biomarker for TNBC. In the present study, we investigated whether LINC00299 methylation is associated with TNBC in a prospective nested breast cancer case–control study within the Generations Study. Methylation at cg06588802 in LINC00299 was measured in 154 TNBC cases and 159 breast cancer-free matched controls using MethyLight droplet digital PCR. To assess the association between methylation level and TNBC risk, logistic regression was used to calculate odd ratios and 95% confidence intervals, adjusted for smoking status. We found no evidence for association between methylation levels and TNBC overall (P = 0.062). Subgroup analysis according to age at diagnosis and age at blood draw revealed increased methylation levels in TNBC cases compared with controls in the young age groups [age 26–52 (P = 0.0025) and age 22–46 (P = 0.001), respectively]. Our results suggest a potential association of LINC00299 hypermethylation with TNBC in young women.

Triple-negative breast cancer (TNBC) is an aggressive breast cancer subtype accounting for 15% of breast cancer in women of Caucasian descent1. It is defined by lack of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression. TNBC is associated with younger age at diagnosis, higher tumor grade, and advanced disease stage and is associated with an increased risk of recurrence and worse survival relative to other subtypes2. The absence of expression of the three receptors strongly reduces targeted treatment options and as such, there is an urgent need to identify novel targets for treatment3,4 or improve early detection.

Aberrant DNA methylation is reported in tumor tissue of many cancers including breast cancer5–6. Similar alterations are detectable in peripheral blood leukocyte (PBL) DNA from cancer patients implying that blood-based DNA methylation markers may be of clinical value for early detection and risk stratification7,8. In breast cancer, several global and gene-specific DNA methylation studies have been performed on PBL-derived DNA1. Global DNA methylation measures have yielded inconsistent findings5,10. There is evidence that local hypermethylation at the BRCA111,12 gene promoter and hypermethylation at the ATM gene body13,14 in PBL DNA is more frequent in breast cancer cases compared with controls. Hypermethylation of the BRCA1 promoter in PBL DNA was also associated with a greater risk of TNBC compared to other breast subtypes, indicating its application as a novel methylation biomarker of increased TNBC susceptibility15. In another study hypermethylation of DOK7 in whole blood DNA was proposed as a powerful epigenetic blood-based biomarker for TNBC16.

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Using a retrospective study design, we recently identified and validated a region within the long noncoding RNA 299 (LINC00299) gene that showed a higher methylation (3% in the discovery set and 2% in the validation set) in PBL DNA from TNBC patients compared with healthy controls, suggesting that this may be a biomarker for TNBC. The hypermethylated region is located in a putative regulatory region of the LINC00299 gene, the function of which is unknown.

In the present study, we tested whether LINC00299 methylation level is associated with TNBC using a prospective study design. Methylation was measured in PBL DNA from 154 TNBC cases and 159 matched controls in a nested case–control study within the prospective Generations Study (GS) cohort using droplet digital PCR (ddPCR).

### Results

Methylation at cg06588802 in LINC00299 was measured in peripheral blood DNA of 154 TNBC cases and 159 matched controls from the study. Selected characteristics of the study participants are shown in Table 1.

The mean methylation levels were higher in TNBC cases compared with controls. However, this difference did not reach statistical significance ($P = 0.062$). In analyses stratified by age, women in the lowest tertile of age at diagnosis (26–52) and age at blood draw (22–46) had statistically significant higher mean methylation levels in cases compared with controls [$P = 0.0025$ and $P = 0.0010$, respectively; (post-hoc power > 90% in both instances); Table 2]. No significant differences between cases and controls were detected for women in the second and third age tertiles.

### Table 1.

Characteristics of triple negative breast cancer cases and controls [matched on year of study entry, age at entry, days blood was in the post before processing, and cancer-free years in the study (time at risk)]. TNBC, triple negative breast cancer. *Including twelve participants with prior cancer (that is not breast cancer).

| Characteristic                        | Cases n (%) | Controls n (%) |
|--------------------------------------|-------------|----------------|
| Total†                               | 154         | 159            |
| Age at study entry [mean (range), years]| 52.1 (24–80) | 52.1 (22–81) |
| Year of study entry                  | 2004–2010   | 2004–2009      |
| Age at blood draw [mean (range), years]| 52.1 (24–80) | 52.2 (22–81) |
| Age at diagnosis date [mean (range), years]| 56.7 (26–87) | –              |
| Blood draw to diagnosis [mean (range), years]| 4.6 (0–10) | –              |
| Days blood in post                   |             |                |
| 0–1                                  | 116 (75.3)  | 120 (75.5)     |
| 2                                    | 11 (7.1)    | 11 (6.9)       |
| 3+                                   | 27 (17.5)   | 28 (17.6)      |

### Table 2.

Difference in methylation levels for triple negative breast cancer cases versus controls: matched analysis (analysis of variance), stratified by age (tertiles). SD, standard deviation; df, degrees of freedom. *Controls matched on year of study entry, age at study entry, days blood in post before processing, and cancer-free years (time at risk). †Heterogeneity test: tests if the difference between cases and controls varies by age.

| Strata                        | Cases | Controls | Matched analysisº |
|-------------------------------|-------|----------|--------------------|
|                               | n     | Mean methylation level (SD) | n     | Mean methylation level (SD) | P value |
| Age at diagnosis              |       |          |                    |       |          |        |
| 26–52                         | 50    | 0.453 (0.036) | 51    | 0.423 (0.043) | 0.0025 |
| 53–62                         | 53    | 0.433 (0.044) | 56    | 0.429 (0.053) | 0.76  |
| 63+                           | 51    | 0.404 (0.059) | 52    | 0.405 (0.056) | 0.94  |
| Heterogeneity (df = 3)º       |       |          |                    |       |          | 0.025 |
| Age at blood draw             |       |          |                    |       |          |        |
| 22–46                         | 48    | 0.454 (0.036) | 52    | 0.427 (0.042) | 0.0010 |
| 47–58                         | 59    | 0.429 (0.049) | 58    | 0.422 (0.055) | 0.46  |
| 59+                           | 47    | 0.406 (0.056) | 49    | 0.410 (0.057) | 0.54  |
| Heterogeneity (df = 3)º       |       |          |                    |       |          | 0.0079 |
had a worse survival than their older (>50 years) counterparts. It is possible that regulatory functions of the three-dimensional chromatin structure showed physical interactions between the genomic region of LINC00299 and its functions. This suggests a potential function of LINC00299 in the development and function of specific immune cells through a variety of mechanisms. NAs have recently emerged as important regulators of gene expression in various cell types. They control the development and function of specific immune cells through a variety of mechanisms. The CpG site cg06588802 is located at the chromosomal region 2p25.1 within the LINC00299 gene. LncRNAs have recently emerged as important regulators of gene expression in various cell types. They control the development and function of specific immune cells through a variety of mechanisms. The hypermethylated region in the LINC00299 gene is evolutionarily conserved and overlaps with several enhancer regions suggesting its possible regulatory functions. Data of the three-dimensional chromatin structure showed physical interactions between the genomic region of LINC00299 and the ID2 gene promoter, a protein with important immune functions. This suggests a potential function of LINC00299 in the regulation of specific immune cells, which needs to be elucidated in functional studies. Further, based on expression data from The Cancer Genome Atlas, ER- and PR-negative breast cancer patients with high LINC00299 expression had a better survival than those with low expression.

In conclusion, though no association between LINC00299 methylation and TNBC overall was observed, our findings suggest that LINC00299 hypermethylation in prediagnostic PBL DNA may be associated with TNBC in young women. If replicated in larger studies, LINC00299 hypermethylation may be of clinical value as a biomarker for early-detection of TNBC in young women.

Table 3. Odds ratio of triple negative breast cancer in relation to methylation levels at cg06588802 in LINC00299, all ages. OR odds ratio, CI confidence interval. *Conditional matched analysis [year of entry to study, age at entry, days blood in post before processing, and cancer-free years (time at risk)], adjusted for smoking status. **Including twelve participants with prior cancer (that is not breast cancer).
Methods

Study population. Study participants were selected from the GS, a long-term prospective breast cancer cohort study focused on potential etiological factors for breast cancer in women in the UK, with blood samples collected at recruitment\(^5\). The study has been approved, under the procedures for national medical research studies, by the South-East Multi-Centre Research Ethics Committee.

The present study selected 161 TNBC cases and matched controls of Caucasian ethnicity for methylation assay. Cases were women who were diagnosed with a first triple-negative invasive or in situ primary breast cancer after study entry. Controls were women with no diagnosis of breast cancer. Individual controls were selected for each case, matched on age (5-year categories), year of study entry (\(\leq 2005, 2006, 2007, \geq 2008\)), the number of days the blood had been in the post (0–1, 2, ≥3), and cancer-free years (time at risk). After updating data files prior to statistical analysis, four cases were subsequently found to have provided blood samples after diagnosis of TNBC and therefore were excluded from the analysis. Further, three cases and two controls had missing methylation data. Hence, nine women (two cases/seven controls) were no longer in a matched pair; these nine women were re-allocated to a matched set with the same age, study entry year, days blood in post grouping, but different cancer free time (thus allowing the inclusion of these women in the matched analysis). In total, the study included 154 cases (diagnosed with a first triple-negative invasive (n = 149) or in situ (n = 5) primary breast cancer after study entry) and 159 controls.

Methylation analysis. DNA samples were extracted from buffy coats using DNA Blood Mini Kits (Qiagen, Hilden, Germany). Two hundred ng of DNA were bisulfite-converted using EpiTect Fast 96 Bisulfite Conversion Kit according to the manufacturer’s protocol (Qiagen). DNA methylation analysis at cg06588802 in LINC00299 was performed using MethyLight droplet digital PCR (ddPCR) as previously described\(^7\). In brief, methylation ratios were defined by locus-specific primers using TaqMan assays. The C-LESS-C1 assay was used as internal control for normalization. Each reaction was performed in a final volume of 20 μl containing 10 μl ddPCR Supermix for Probes (No dUTP) (Bio-Rad, Hercules, CA, USA), 900 nM forward and reverse primers, 250 nM probe, 2 μl bisulfite-converted DNA template, and 6 μl nuclease-free double distilled H₂O. All ddPCR steps were performed according to the manufacturer’s protocols (Bio-Rad). Cycling was at 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s and 59 °C for 60 s, and a final step at 98 °C for 10 min. To ensure equal conditions for cases and controls during all ddPCR steps, case–control pairs were allocated on the same cartridge of the assay to minimize batch effects. Duplicates and fully methylated and unmethylated controls were used on each ddPCR plate for quality control.

Statistical analysis. Analysis of variance was used to assess the difference in methylation levels between TNBC cases and matched controls (i.e. with implicit adjustment for the matching factors).

Stratified analysis (tertiles) was performed by age at diagnosis (for controls this was the matched case’s age at diagnosis) and age at blood draw.

For the association between methylation level and TNBC risk, conditional logistic regression was used to calculate odd ratios (ORs) and 95% confidence intervals (CIs). We adjusted for smoking status (at recruitment: never, ex-smoker, current) as LINC00299 methylation level is changed in response to cigarette smoking\(^8\).

All P values were two-sided, with a P value of 0.05 considered statistically significant. Stata/IC version 14.2 was used for all analyses\(^9\).

Ethical approval. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study has been approved by the South-East Multi-Centre Research Ethics Committee.

Informed consent. Informed consent was obtained from all individual participants included in the study.

Data availability

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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**Author contributions**

U.H. and M.M. contributed to the study conception and design. Material preparation, data collection and molecular analyses were performed by U.H., M.M., K.T., O.F., N.B., M.J.S., and A.J.S. Statistical analysis was performed by M.J. The first draft of the manuscript was written by U.H. and M.M. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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