IGF2R genetic variants, circulating IGF2 concentrations and colon cancer risk in African Americans and Whites

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Abstract. The Mannose 6 Phosphate/Insulin-like Growth Factor Receptor-2 (IGF2R) encodes a type-1 membrane protein that modulates availability of the potent mitogen, IGF2. We evaluated the associations between IGF2R non-synonymous genetic variants (c.5002G>A, Gly1619Arg(rs629849), and c.901C>G, Leu252Val(rs8191754)), circulating IGF2 levels, and colon cancer (CC) risk among African American and White participants enrolled in the North Carolina Colon Cancer Study (NCCCS). Generalized linear models were used to compare circulating levels of IGF2 among 298 African American and 518 White controls. Logistic regression models were used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the association of IGF2R genetic variants and CC risk. Women homozygous for the IGF2R c.5002 G>A allele, had higher mean levels of circulating IGF2, 828 (SD = 321) ng/ml compared to non-carriers, 595 (SD = 217) ng/ml (p-value = 0.01). This pattern was not apparent in individuals homozygous for the IGF2R c.901 C>G variant. Whites homozygous for the IGF2R c.901 C>G variant trended towards a higher risk of CC, OR = 2.2 [95% CI(0.9–5.4)], whereas carrying the IGF2R c.5002 G>A variant was not associated with CC risk. Our findings support the hypothesis that being homozygous for the IGF2R c.901 C>G variant may increase cancer risk, the mechanism may not involve modulation of circulating IGF2.

Keywords: IGF2R polymorphism, colon cancer, IGF2 concentration

1. Introduction

Colon cancer is the third most commonly diagnosed cancer and the second leading cause of cancer death in the US \cite{1,2}. Over 106,600 new cases are diagnosed each year and 55,170 individuals die from this disease in the U.S. alone \cite{1}. The highest incidence and mortality are among African Americans and individuals residing in the southeastern seaboard of the United States \cite{2}. Whereas reasons for higher mortality can be attributed to poorer access to screening resulting in later stage at diagnosis among African Americans compared to Whites \cite{3}, reasons for higher incidence are unclear. The interaction of genetic and environmental factors has been hypothesized to underlie racial differ-
rances for some time [4], however, the causative genetic loci are still unknown. Genetic variation in genes encoding insulin and insulin-like growth factors (IGF) have been evaluated in relation to CC risk although the focus has been on IGF1 [4–10]. Few studies have evaluated IGF2, also a potent mitogenic growth factor, in circulation at higher physiologic concentrations than IGF1; IGF2 also has been associated with several malignancies [11].

The Mannose 6 Phosphate/Insulin-like Growth Factor 2 Receptor (IGF2R) encodes a ∼300 kDa multi-functional type 1 membrane protein recently identified as a tumor suppressor [12,13]. Although IGF2R regulates transport of lysosomal proteases and activates latent transforming growth factor β1, a potent growth inhibitor [14], this receptor also sequesters free IGF2 ligand, internalizing it for intracellular trafficking to the lysosomes for degradation [13–18], thereby modulating IGF2 availability and its mitogenic activity. In the mouse, loss of IGF2R function results in disproportionate embryonic overgrowth and perinatal death [19,20]. In human cells, loss of IGF2R function has been associated with the development of cancers of the breast, lung, liver and ovary [21,22].

More than 1,700 genetic variants of IGR2R have been reported to the NCBI database to date. In functional studies, Killian and Jirtle et al. [13] described six of these SNPs in the coding region of IGF2R, of which three are non-synonymous [c.6206 A>G, Asn2020Ser; c.901C>G, Leu252Val and c.5002G>A Gly1619Arg]. The effects of these genetic polymorphism on IGF2R function are only now beginning to be elucidated in physiological studies [15,23]. Despite their functional significance, these genetic variants are generally not interrogated in Genome-wide Association Studies (GWAS), because the commonly used Affymetrix 100K [24] and the Illumina Hap300 [25] chips do not include these rare alleles. Herein we evaluate two of the three non-synonymous IGR2R variants, c.901 C>G and c.5002 G>A, that have a minor allele population frequency of more than 5%, their association with higher IGF2 circulating levels, and colon cancer risk in African Americans and Whites.

2. Methods

2.1. Study participants

Study participants were recruited as part of the North Carolina Colon Cancer Study (NCCCS) and detailed accrual methods have been described in earlier reports [26]. Briefly, the NCCCS is a population-based, case-control study based in 33 contiguous rural and urban counties in central North Carolina comprising 39% African Americans (294 cases and 437 controls) and 61% Whites (349 of them cases and 611 controls). Cases of histologically confirmed invasive adenocarcinoma of the colon aged 40–85 diagnosed between July 1, 1996 and June 30, 2000 were identified using the rapid case ascertainment system administered by the North Carolina Cancer Registry. Because the sampling frame for < 65 year age group was the drivers’ license roster, cases aged < 65 years had to have a North Carolina’s driver’s license or state identification cards. To oversample African Americans and women, and also maintain analytic flexibility, a randomized recruitment strategy [27,28] was employed; adjusted for in statistical analyses using a sampling fraction determined a priori. Controls under age 65 were sampled from the North Carolina’s Division of Motor Vehicles driver’s license list, matched by race and sex and frequency-matched by 5-year age group. Controls aged 65 years and older were frequency-matched to cases using the Medicare-eligible beneficiaries’ roster. We were able to contact 86% of cases identified through the registry, and (84%), agreed to participate, an overall response rate of 72%, higher in Whites (76%) than African Americans (62%). Ninety-percent of controls identified were successfully contacted and of these, 62%, agreed to participate. Individuals who refused to participate or could not be reached were slightly younger than those who participated. The study was approved by the IRB at the University of North Carolina and all patients gave informed consent.

2.2. Data collection

Questionnaire data were collected using a standardized questionnaire by trained interviewers in person as previously described [26]. Information solicited included socio-demographic characteristics such as age, sex, marital status, education and household income; anthropometric measurements, including estimated weight one year prior to the interview and weight at interview, family history of cancer and personal medical history, as well as lifestyle factors that included physical activity, occupational history, smoking habits, and caloric intake. Weight, circumference of the waist and hip, and height were also measured at the time of interview. Information on nutrition was obtained using a version of the Block semi-quantitative ques-
The questionnaire [29,30] modified to include some regional foods common in NC with a one year reference to fully account for seasonality. The questionnaire has been validated [31]. Information on non-steroidal anti-inflammatory drugs (NSAID) use was based on an affirmative response to a battery of questions about frequency of intake of several pharmaceutical products by name.

2.3. Collection and processing of blood specimens

Blood samples were collected from consenting participants at the time of the interview. All blood specimens were received by the laboratory within 24 hours, where buffy coat and plasma were separated, aliquoted and stored at −80°C. Eighty six percent of cases (n = 529) and 83% of controls (n = 836) who agreed to participate also agreed to provide peripheral blood samples for DNA and protein analyses. Participants who gave blood were more likely to be male, white, and to have never smoked compared to those who did not donate blood samples. To evaluate whether individuals who provided blood samples systematically differed from those who declined, we compared anthropometric measurements, NSAID use and stage at diagnosis of cases and found blood donors and non-donors to be comparable (data not shown).

2.4. Enzyme linked immunosorbent assays (ELISA) for IGF2

Plasma IGF2 was measured after acid-ethanol extraction to remove IGFBPs using reagents from Diagnostic Systems Laboratory (Webster, Texas), according to manufacturer’s protocol. These analyses were measured in duplicates on stored plasma samples and laboratory personnel were blinded to the case or control status of samples. IGF2 levels were measured in 805 and 522 blood samples of the controls and cases, respectively. Each assay batch included positive controls and, masked samples derived from pooled plasma specimens were received by the laboratory within 24 hours, where buffy coat and plasma were separated, aliquoted and stored at −80°C. Eighty six percent of cases (n = 529) and 83% of controls (n = 836) who agreed to participate also agreed to provide peripheral blood samples for DNA and protein analyses. Participants who gave blood were more likely to be male, white, and to have never smoked compared to those who did not donate blood samples. To evaluate whether individuals who provided blood samples systematically differed from those who declined, we compared anthropometric measurements, NSAID use and stage at diagnosis of cases and found blood donors and non-donors to be comparable (data not shown).

2.5. Genotyping

Genomic DNA was extracted from buffy coat using the PureGene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN). Genotyping was done using the 5’ exonuclease (Taqman) assay. Primers for the IGF2R c.5002 G>A (rs629849) and c.901 C>G (rs8191754) variants were designed by Applied Biosystems (Foster City, CA). For c.901 C>G, the forward primer was 5’-CTA AGG GTA CTG TGA TTA TCA CTC-3’ and the reverse primer was 5’-GAA AGT CAG GTC CTG CCT GCT GGA G-3’. For the c.5002 G>A, the forward primer was 5’-GAA ATT GAT GGT CCT GAC TTG CG-3’ and the reverse primer was 5’-GCA CTG GAG ATG CAC TTC TCC-3’. For quality control, a randomly selected 10% of samples were run in duplicate and genotyping was conducted without the knowledge of case status. Undetermined genotypes were excluded from analyses and constituted 1% and 0.7% of the total population for the IGF2R c.901 C>G and IGF2R c.5002 G>A variants, respectively. The average genotype concordance rate for duplicate samples was > 98%.

2.6. Statistical analyses

We used chi-square tests to evaluate deviation from Hardy-Weinberg equilibrium (HWE) of IGF2R c.5002 G>A and IGF2R c.901 C>G genotypes among controls for Whites and African Americas separately. We used t-tests and analysis of variance, to evaluate mean differences in IGF2 levels between individuals who carried one or two of the minor alleles (‘CG’ or ‘GG’ for IGF2R c.901 C>G; ‘GA’ or ‘AA’ for IGF2R c.5002 G>A) and those homozygous for the common allele (‘CC’ or ‘GG’, for IGF2R c.901 C>G and IGF2R c.5002 G>A, respectively), adjusting for sex, age and race, since participants varied by these characteristics. Unconditional logistic regression models were used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the association between carrying these genotypes and risk of colon cancer risk, again comparing carriers of at least one of the minor alleles to non-carriers, adjusting for potential confounding factors. Matching variables, age, sex and race were included in all models estimating main effects as was the sampling fraction [26–28]. Effect modification by body mass index (BMI = kg/m²), cigarette smokers (ever, vs. never), caloric intake (1,800 or more vs. less than 1,800 calories per day), circumference of the waist (< 102 cm vs. ≥ 102 cm), use of NSAIDS (NSAID use for ≥ 15 months vs. < 15 months in preceding diagnosis for cases and interview for controls), was evaluated using stratified analyses and cross-product terms of the genotype and potential effect modifier. We also assessed potential confounding by socioeconomic factors including household in-
come and education, as well as lifestyle factors, including daily average caloric intake, cigarette smoking and frequency of alcohol intake. These factors did not change the \( \beta \)-coefficient for the association between genotypes evaluated and colon cancer by \( \geq 10\% \), and were thus not retained in final models. Statistical models were developed for African Americans and Whites separately and then combined. We used SAS version 9.1 (SAS, Cary, NC, USA) for all statistical analyses.

### 3. Results

Characteristics of study participants have been previously described in several reports [26,32,33]. Table 1 summarizes the distribution of characteristics of study participants. Although minor gender variations were noted, in general, when compared to controls, cases were older, reported higher daily average caloric intake, had a higher BMI one year prior to interview, a larger waist circumference at interview, and were less likely to report intake of NSAIDs. However, cases did not significantly differ from controls with respect to education, alcohol consumption and a history of cigarette smoking.

To gain insights into the functional significance of \( IGF2R \) genetic variants, we evaluated whether individuals who carry the \( IGF2R \) c.5002 G>A and \( IGF2R \) c.901 C>G variants had higher average circulating levels of the IGF2 ligand among otherwise healthy controls (Table 2). We found that individuals homozygous for the \( IGF2R \) c.5002 G>A variant had higher mean levels of circulating IGF2, 593 (SD = 204) ng/ml compared to non-carriers, 563 (SD = 206) ng/ml (p-value = 0.06). These differences in mean IGF2 levels were most pronounced among women (p-value = 0.01). Notably, a gene-dose response trend was apparent in both African Americans and Whites (p for trend = 0.003). Adjusting for age, sex and race did not alter these findings.

![Image](image.png)

Table 1

Distribution of characteristics of cases and controls in the North Carolina Colon Cancer Study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>White men (n = 466)</th>
<th>White women (n = 371)</th>
<th>Black men (n = 263)</th>
<th>Black women (n = 294)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Case (n %)</td>
<td>Control (n %)</td>
<td>Case (n %)</td>
<td>Control (n %)</td>
</tr>
<tr>
<td>Age Group &lt; 65</td>
<td>75 (44)</td>
<td>111 (37)</td>
<td>56 (41)</td>
<td>81 (35)</td>
</tr>
<tr>
<td>Age Group ( \geq 65 )</td>
<td>94 (56)</td>
<td>186 (63)</td>
<td>81 (59)</td>
<td>153 (65)</td>
</tr>
<tr>
<td>Education Level &lt; HS grad</td>
<td>50 (30)</td>
<td>67 (23)</td>
<td>32 (23)</td>
<td>46 (20)</td>
</tr>
<tr>
<td>Education Level HS grad, college</td>
<td>74 (44)</td>
<td>137 (46)</td>
<td>79 (58)</td>
<td>136 (52)</td>
</tr>
<tr>
<td>Education Level College grad or more</td>
<td>44 (26)</td>
<td>92 (31)</td>
<td>26 (19)</td>
<td>52 (22)</td>
</tr>
<tr>
<td>NSAID use in past year Yes</td>
<td>81 (48)</td>
<td>175 (59)</td>
<td>48 (35)</td>
<td>122 (52)</td>
</tr>
<tr>
<td>NSAID use in past year No</td>
<td>88 (52)</td>
<td>122 (41)</td>
<td>89 (65)</td>
<td>112 (48)</td>
</tr>
<tr>
<td>BMI 1 year ago 18.5–25 (Normal)</td>
<td>29 (17)</td>
<td>76 (26)</td>
<td>54 (41)</td>
<td>80 (36)</td>
</tr>
<tr>
<td>BMI 1 year ago 25–30 (Overweight)</td>
<td>79 (48)</td>
<td>143 (49)</td>
<td>46 (35)</td>
<td>77 (34)</td>
</tr>
<tr>
<td>BMI 1 year ago 30+ (Obese)</td>
<td>58 (35)</td>
<td>70 (24)</td>
<td>31 (24)</td>
<td>68 (30)</td>
</tr>
<tr>
<td>Regular Multivitamin User Yes</td>
<td>64 (39)</td>
<td>141 (51)</td>
<td>74 (56)</td>
<td>136 (61)</td>
</tr>
<tr>
<td>Regular Multivitamin User No</td>
<td>100 (61)</td>
<td>134 (49)</td>
<td>59 (44)</td>
<td>86 (39)</td>
</tr>
<tr>
<td>Smoking Status Current</td>
<td>21 (13)</td>
<td>55 (19)</td>
<td>20 (15)</td>
<td>28 (12)</td>
</tr>
<tr>
<td>Smoking Status Former</td>
<td>111 (66)</td>
<td>158 (53)</td>
<td>50 (36)</td>
<td>81 (35)</td>
</tr>
<tr>
<td>Smoking Status Never</td>
<td>35 (21)</td>
<td>83 (28)</td>
<td>67 (49)</td>
<td>125 (53)</td>
</tr>
<tr>
<td>Alcohol Use Yes</td>
<td>74 (45)</td>
<td>138 (48)</td>
<td>47 (35)</td>
<td>70 (31)</td>
</tr>
<tr>
<td>Alcohol Use No</td>
<td>90 (55)</td>
<td>148 (52)</td>
<td>88 (65)</td>
<td>157 (69)</td>
</tr>
<tr>
<td>Calories per day ( \leq 1,800 )</td>
<td>44 (27)</td>
<td>102 (35)</td>
<td>88 (64)</td>
<td>175 (75)</td>
</tr>
<tr>
<td>Calories per day &gt; 1,800</td>
<td>120 (73)</td>
<td>192 (65)</td>
<td>49 (36)</td>
<td>58 (25)</td>
</tr>
</tbody>
</table>
separately and also in men and women. The patterns persisted although the difference was most pronounced in women (p-value = 0.06). We found no evidence for higher IGF2 levels in individuals who carry both IGF2R c.901 C>G and the IGF2R c.5002 G>A genetic variants.

Table 3 shows the distribution of genetic variants IGF2R c.901 C>G and IGF2R c.5002 G>A, and the ORs and 95% CIs for the associations between carrying the variant alleles and risk of colon cancer among all participants and separately by race/ethnicity. There was no evidence that genotype frequencies in the IGF2R c.901 and C.5002 variants means and standard deviations of plasma IGF2 levels (ng/ml) in n = 805 controls carrying the IGF2R c.901C>G, IGF2R c.5002G>A, and the IGF2R c.901 and c.5002 variants in African Americans and Whites.

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4. Discussion

We present the first population-based, epidemiologic evidence that otherwise healthy individuals who carry the non-synonymous IGF2R c.901 C>G, Leu252Val or c.5002 G>A Gly1619Arg genetic variants have higher average IGF2 levels. Gene-dose response trends were most apparent in individuals homozygous for the IGF2R c.5002 G>A and colon cancer risk in all participants, OR = 1.9 [95% CI(0.9–4.1)] was observed. This association appeared to be primarily among Whites, OR = 2.2 [95% CI(0.9–5.4)] and not African Americans, OR = 1.4 [95% CI(0.3–6.6)]. However, the number of individuals homozygous for this genotype in African Americans was small. This association did not vary by NSAID use, BMI, waist circumference, cigarette smoking and daily caloric intake, (data not shown). We found no evidence for an association between being homozygous for the IGF2R c.5002 G>A and colon cancer risk among Whites, OR = 0.9 [95% CI(0.7–1.3)], and African Americans, OR = 0.9 [95% CI(0.4–1.8)]. We also evaluated whether the accumulation of the two genetic variants increased colon cancer risk, and no such synergistic association was found, (data not shown).

Genome-wide Association Studies (GWAS), because the commonly used Affymetrix 100K [24] and the Illumina Hap300 [25] chips do not include these genetic variants.

Our findings that carrying the IGF2R c.5002 G>A is associated with elevated IGF2 levels are consistent with previous studies showing that variant IGF2R c.5002 G>A disrupts IGF2 ligand binding functions within IGF2R domain 11 [36,37]. However, a more recent NMR study showed no association between this polymorphism and IGF2 binding [15], and crystallography data showed that Gly1619Arg does not lie within the IGF2 binding site [16]. This polymorphism though, could be affecting splice site choice and exon skipping either directly or through linkage disequilibrium (LD) with other SNPs located in intronic sequences [15]. A recent study found a threefold increased risk of advanced oral squamous cell carcinoma in individuals carrying the IGF2R c.5002 variant Gly1619Arg A-allele compared to the wild type or G-allele [38]. IGF2R variant haplotypes were also associated with increased pancreatic cancer risk [39]. IGF2 circulating levels were not reported in these studies. Zhao et al. [40], reported that very high levels of circulating IGF2 are found in advanced colorectal cancer, however Reneman et al. [41] found an association between serum IGF2 and early colon cancer stages.

IGF2R binds and internalizes IGF2 maintaining appropriate levels of IGF2 locally and in circulation. When this equilibrium is disrupted either by loss of IGF2R function [18–20] or IGF2 loss of imprinting [42, 43], increased growth occurs in human and murine tumors [44,45]. However, the exact mechanism and its association to IGF2R polymorphisms is still unknown. Evidence based on published data suggests that even if IGF2R c.5002 G>A has no effect on function, it is in strong LD with a causative mutation. However, informatics suggest that IGF2R c.5002 G>A may have a regulatory effect at the RNA level. IGF2R c.5002 G>A sits in possible binding sites for multiple miRNAs, with the G and A alleles creating sequences complementary to different miRNAs. In addition, splice site predictions shows that the A- allele may create a splice acceptor site, as well as eliminate an enhancer motif for Serine/Arginine (SR) protein binding [46]. Recent results have indicated that IGF2R exon 34, which contains c.5002 G>A, is affected by alternative splicing, and is also contained in an antisense transcript [47]. The role of IGF2R c.5002 G>A or linked polymorphisms in splice site choice, as well as antisense expression or function, have yet to be determined.
We observed a different population distribution for the IGF2R c.5002 G>A variant when we stratified by sex and race: HWE deviations were more common in women ($p = 0.003$) and in African Americans ($p = 0.02$). HWE deviations in black women could have been due, at least in part to small sample size of African Americans ($n = 20$) carrying the IGF2R c.5002 G>A allele, compared to Whites ($n = 119$); genotyping errors were uncommon. Furthermore, IGF2R c.5002 G>A is a rare allele in linkage disequilibrium (LD) with a potentially functional mutation. The level of LD is influenced by the rate of mutation and population structure and it is known to influence HWE [48]. However, the association of variant IGF2R c.5002 G>A and IGF2 levels in women is strong ($p = 0.01$) and unlikely to be affected by the deviation from HWE, although further studies with a larger sample of women are required.

Although we also found a correlation between higher levels of IGF2 and carriers of the IGF2R c.901 C>G variant in healthy women, the molecular mechanisms underlying the suggested association are unclear and may differ from those related to the IGF2R c.5002 G>A variant. The amino acid variant c.901 Leu252Val is located in repeat domain 3 and this domain is involved in binding M6P moieties on other proteins [12]. Thus, IGF2R c.901 C>G may also alter function by changing the affinity for M6P bearing ligands which in turn may alter protein trafficking, with a consequent increase in the IGF2 ligand [50,51]. In predominantly white populations, carrying the IGF2R c.901 C>G has been associated with another gastrointestinal malignancy [52] and a tall stature [53]. Tall stature has been associated with colon cancer risk [54], and elevated IGF2 levels [55]. In the current data set, colon cancer risk was that women homozygous for the IGF2R c.901 GG variant, and intriguingly, four of the five women were colon cancer cases compared to seven of fifteen men.

Data from publically available databases including HapMap and SNP500, suggest that the relative population frequency of IGF2R c.901 C>G and IGF2R c.5002 G>A do not parallel the higher incidence of colon cancer in African Americans. The prevalence of IGF2R c.901 C>G is similar in Whites (25%) and African Americans (22%), yet the risk of colon cancer associated with carrying this allele differs markedly between the two groups. In contrast, IGF2R c.5002 G>A is rare in African Americans (13%) and common in Whites (36%). This may suggest that while these genetic variants may contribute to modulating IGF2 concentrations via IGF2R binding, other mechanisms including deregulation of genomic imprinting may also play a role. Relaxation of imprinting controls leading to biallelic expression of IGF2 may also increase circulating IGF2 ligand directly and this may be important since the population frequency of IGF2 loss of imprinting could be as high as 10% to 20% in the population [51]. IGF2 loss of imprinting has been associated with a five-fold increase in colon cancer risk [34,35]. When taken together, IGF2R c.5002 G>A and c.901 C>G polymorphisms may exert their effects in a multifaceted manner, among them, by modulating IGF2 in circulation.

A major strength of this study is that it is one of the largest population-based case-control studies with the largest number of African Americans included, enabling evaluation of genetic effects on both colon cancer risk and on IGF2 protein concentrations in African Americans and Whites. Although this is not a genome-wide study and we cannot exclude the influence of other genes or other genetic variants in the insulin growth factor or related pathways, we evaluated genetic variants known to be amino acid-changing and found positive associations between carrying either variant and IGF2 protein levels. Despite its size, a limitation of this study is that the sample size limited our ability to adequately evaluate race-specific associations of these relatively rare alleles, as well as gene-environment interaction effects on colon cancer risk within race/ethnic group-specific associations. Departure from HWE may have been due, at least in part, to small sample size and rare allele frequencies of variant IGF2R c.5002 G>A in African American women.

In summary, we provide the first epidemiologic evidence that women homozygous for the IGF2R c.5002 G>A genetic variant have, on average, higher circulating levels of the mitogen IGF2. IGF2 is associated with increased risk of developing colorectal neoplasia. Our data also suggest that carrying the non-synonymous IGF2R c.901 C>G may be associated with increased colon cancer risk, although this association may not be via modulation of the ligand. Future studies in a larger sample of individuals carrying these rare allele variants are required to evaluate IGF2 levels and CC risk associations.

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