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Transforming growth factor β receptor I polyalanine repeat polymorphism does not increase ovarian cancer risk

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Abstract

Objectives. It has been suggested that the 6A allele of the type I TGFβ receptor (*TGFβR1*) polyalanine repeat tract polymorphism may increase susceptibility to various types of cancer including ovarian cancer.

Methods. The *TGFβR1* polyalanine polymorphism was genotyped in 588 ovarian cancer cases and 614 controls from a population-based case-control study in North Carolina.

Results. Significant racial differences in the frequency of the 6A allele were observed between Caucasian (10.7%) and African-American (2.4%) controls ($P < 0.001$). One or two copies of the 6A allele of the *TGFβR1* polyalanine polymorphism was carried by 18% of all controls and 19% of cases, and there was no association with ovarian cancer risk (OR = 1.07, 95% CI 0.80–1.44). The odds ratio for 6A homozygotes was 1.81 (95% CI 0.655.06), but these comprised only 0.98% of controls and 1.70% of cases.

Conclusions. The 6A allele of the *TGFβR1* polyalanine polymorphism does not appear to increase ovarian cancer risk. Larger studies would be needed to exclude the possibility that the small fraction of individuals who are 6A homozygotes have an increased risk of ovarian or other cancers.

Keywords: *TGFβRI*; Ovarian cancer; 6A allele; Case-control study

Introduction

The transforming growth factor β (TGFβ) family of peptide growth factors have pleiomorphic effects on cellular signaling, growth, differentiation and apoptosis and are potent negative regulators of cell growth [1–3]. Decreased TGFβ activity due to dysregulation of elements of its associated signal transduction pathways facilitates unrestrained proliferation and a propensity for malignant transformation. Conversely, the TGFβ pathway is upregulated by some agents such as anti-estrogens and retinoids that decrease cancer risk [4]. Thus, inherited or acquired alterations in members of the TGFβ pathway could affect cancer susceptibility and the process of malignant transformation.

TGFβ signaling is initiated by three cell surface receptors, the type I and II serine/threonine kinase receptors [1,5] and the type III betaglycan receptor [6,7]. A repetitive microsatellite sequence in the coding region of the type II receptor is the target for inactivating mutations in some cancers with defective DNA mismatch repair [5]. The type I TGFβ receptor (*TGFβRI*) is not a target of microsatellite instability [5]; however, it has been suggested that a polyalanine repeat polymorphism in TGFβRI increases susceptibility to colorectal and other cancers [8–10]. The most common allele of this polymorphism encodes 9 alanine (9A) amino acid residues. The next most common allele is nine base pairs shorter producing a *TGFβRI* with 6 alanines (6A). Other

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polymorphic alleles with 5, 7, 8, 10, 11 and 12 alanine encoding GCC repeats have been described as well [8,11]. Most prior studies of the *TGFβR1* polyalanine repeat polymorphism have not focused specifically on ovarian cancer, but a meta-analysis of published case-control studies has suggested a protective effect [10]. We sought to confirm this finding in a large population-based case-control study of ovarian cancer performed in North Carolina.

Materials and methods

Case-control study design

Epithelial ovarian cancer cases and controls were enrolled in a continuing population-based case-control study approved by the Duke University IRB. The 588 cases were identified from the North Carolina Central Cancer Registry and included women aged 20–74 with newly diagnosed epithelial ovarian cancer residing in a 48 county region of North Carolina. The ovarian cancer diagnosis was confirmed by the study pathologist. The 614 controls were identified by either random digit dialing or Health Care Financing Administration phone lists. Controls were matched for 5 year age intervals and race (black or non-black) from the same 48 county area of North Carolina. All controls were required to have at least one intact ovary. Both cases and controls participated in an extensive in-home interview conducted by study nurses. Epidemiologic data related to known and suspected ovarian cancer risk factors were collected. Laboratory investigators were blinded to the identity of cases and controls.

DNA extraction

A 30 ml peripheral blood sample was drawn from each woman at the time of the nurse interview. Genomic DNA was extracted from leukocytes using a Puregene DNA Isolation kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's protocol. An aliquot of the stock DNA was diluted to a PCR concentration of 30 ng/μl and the remainder of the stock frozen at −70C. PCR DNA dilutions were arranged in 96 well microtiter plates.

PCR

Genomic DNA was amplified using the Advantage Genomic PCR kit (BD Biosciences) and previously published primer sequences FOR: 5′ CCACAGGCGGTG-GCGGCGGGACCATG3′ and REV: 5′ [12]. 60 ng of DNA was amplified with 1× PCR buffer, 4× dNTPs, 1 μM GC-Melt, 0.5 × Advantage Taq polymerase mix and 10 μM each forward and reverse primer. The forward primer was 5′ labeled with FAM (Sigma) and HPLC purified. Amplification conditions were as follows: 95°, 4 min; 94°, 30 s, 70°, 8 min times 30 cycles.

Genotyping

Fluorescent fragment analysis was performed by the Duke University DNA Analysis core facility. Fluorescently labeled PCR products were diluted 250-fold in sterile water and then run on an Applied Biosystems 3100 Genetic Analyzer with a 50 cm capillary array, POP6 polymer and ROX400HD size standards. Allele peak sizes were assigned to each sample after comparison with sequence verified *TGFβR1* 6A and 9A standards. All samples with 6A alleles

Table 1
Demographic and clinical features of ovarian cancer cases and controls in the North Carolina Ovarian Cancer Study

	Cases (N = 588)		Controls (N = 614)		
Age in years					
Mean (SD)	54.1	(11.5)	54.8	(12.3)	
Median (range)	54	(20–74)	54	(20–75)	
	n	(%)	*n*	(%)	*P* value
Race					
Caucasian	495	(84)	520	(85)	
African-American	77	(13)	83	(14)	
Other	16	(3)	11	(2)	
Menopause status					
Pre/Peri	226	(39)	248	(40)	0.55
Post	361	(61)	366	(60)	
Tubal ligation					
No	443	(75)	403	(66)	<0.001
Yes	144	(25)	211	(34)	
Oral contraceptive use (months)					
None	208	(35)	196	(32)	0.09
≤12	101	(17)	100	(16)	
>12	265	(45)	309	(50)	
User of unknown duration	13	(2)	9	(1)	
Livebirths					
0	123	(21)	81	(13)	<0.001
1	105	(18)	94	(15)	
>1	359	(61)	439	(71)	
Family history of ovarian cancer					
No	562	(96)	596	(97)	0.04
Yes	25	(4)	17	(3)	
Tumor behavior					
Borderline	133	(23)			
Invasive	454	(77)			
Histologic subtype					
Serous	353	(60)			
Endometrioid	71	(12)			
Mucinous	70	(12)			
Clear Cell	37	(6)			
Other	57	(10)			
Stage					
I	208	(35)			
II	42	(7)			
III	310	(53)			
IV	19	(3)			
Unknown	9	(2)			

Odds ratios are age and race adjusted.
1 missing tumor behavior and 5 missing stage.

were verified in a second independent PCR amplification. Further confirmation of allele size was provided by directly digesting the PCR products with BssSI (New England Biolabs). Digested PCR products were resolved on 6% nondenaturing polyacrylamide gels and visualized with ethidium bromide staining. The digest yields a common 66 bp fragment and 44 bp (6A), 47 bp (7A), 50 bp (8A), 53 bp (9A) and 56 bp (10A) variable fragments. Two ovarian cancer cell lines in which the polymorphism had been sequenced were used as positive controls for the 9A/9A (OVCA 433) and 6A/6A (OVCA432) genotypes.

Statistical analysis

The genotype data were tested for Hardy Weinberg Equilibrium using the Chi-square goodness of fit test. Multivariate unconditional logistic regression models, adjusted for age, were used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) for the association between the *TGFβRI* polymorphism and ovarian cancer for all cases as well as for various disease subsets. All calculations were performed using SAS 8.0 (SAS Institute Inc., Cary, NC). The sample size of the combined study provided 80% power to detect an OR of 1.5 or greater with a two-sided type 1 error level of 0.05.

Results

The demographic features, epidemiologic risk factors and pathological characteristics of cases and controls in the North Carolina ovarian cancer study are shown in Table 1. Eighty-four percent of cases and 85% of controls were Caucasian, while 13% of the cases and 14% of controls were African-American. The median age was also similar in each group. The known relationships between various

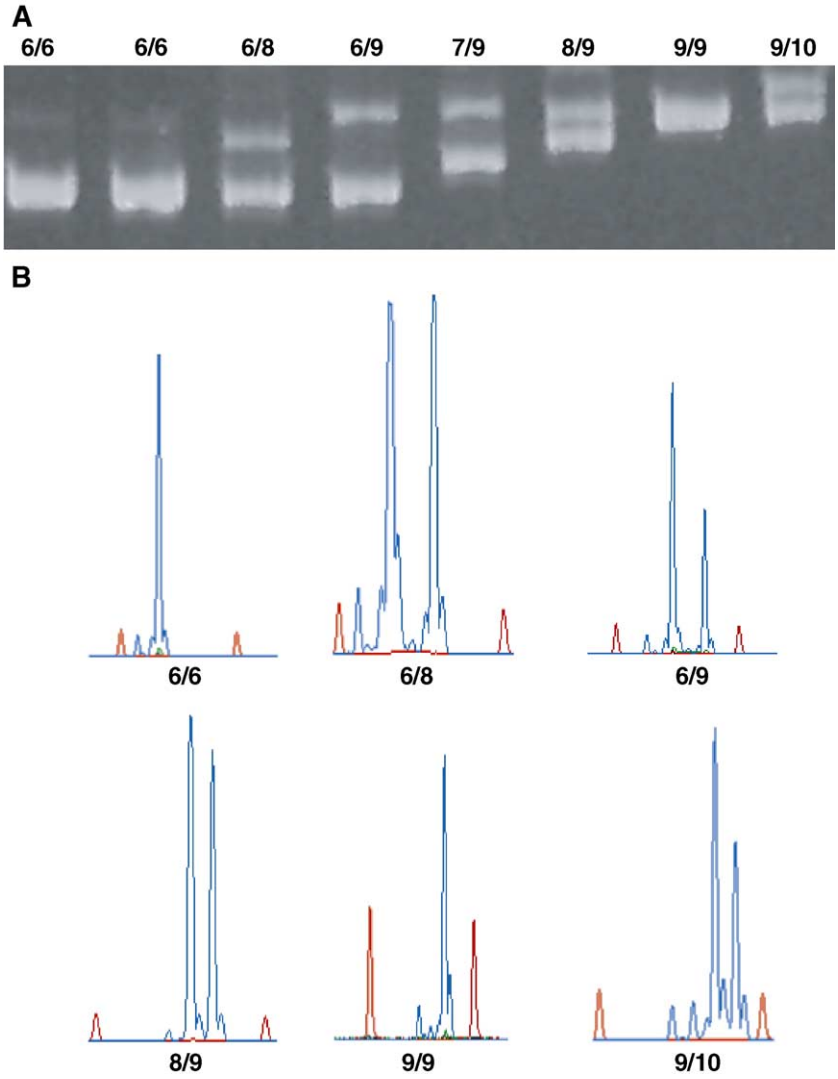

Fig. 1. *TGFβRI* polyalanine repeat polymorphism. (A) Restriction fragment analysis: Lane 1 demonstrates a 6A homozygote positive control (ovarian cancer cell line OVCA 432). Lanes 2–8 represent various alleles seen in North Carolina Ovarian Cancer study subjects. (B) Fluorescent fragment analysis: Homozygous samples (6A/6A and 9A/9A) have single peaks and heterozygous samples two peaks. Small peaks bracketing the sample peaks represent internal size standards.

epidemiologic risk factors and ovarian cancer were observed in this study. Oral contraceptive use, tubal ligation and live births were less frequent among cases compared to controls whereas a family history of ovarian cancer was more common among case versus control subjects. The distribution of cases by tumor behavior (borderline versus invasive), histologic type and stage is also shown in Table 1.

The *TGFβR1* polymorphism was initially examined in PCR products of ovarian cancer cell lines and study subjects using the technique employed in most prior studies (polyacrylamide gel electrophoresis following BssSI digestion) as well as with fluorescent fragment analysis (Fig. 1). Identical results were obtained using both techniques and the polymorphism was subsequently analyzed in all study subjects using fluorescent fragment analysis. All samples reported as 6A/6A or 6A/9A were confirmed using a second independent PCR reaction. The *TGFβR1* polymorphism was genotyped in 1202 subjects including 614 controls and 588 cases. Among controls, the distribution of genotypes was found to be in Hardy Weinberg Equilibrium ($\chi^2 = 0.247$, $P = 0.97$). The overall frequency of the 9A allele was 89.9%, and that of the 6A allele was 9.5%. The combined frequency of other alleles (7A, 8A, 10A) was only 0.6%. Significant racial differences in allele frequencies were observed. Among 520 Caucasian controls, the frequency of the 6A allele was 10.7% compared to only 2.4% in 83 African-American controls ($P < 0.001$). Alleles other than 9A and 6A were seen almost exclusively in African-Americans (3.6% allele frequency) and were rare in Caucasians (0.1%).

Table 2 summarizes the frequency of the *TGFβR1* polyalanine repeat genotypes in the entire study population and in Caucasians and African-Americans. There was no association between the *TGFβR1* 6A genotype and ovarian cancer risk. When 6A heterozygotes and homozygotes were combined and compared to 9A homozygotes, the risk of ovarian cancer risk was 1.07 (95% CI 0.80–1.44). Although the odds ratio for 6A homozygotes alone was 1.81, the frequency of homozygotes was only 0.98% in controls and 1.70% in cases, and the 95% confidence intervals for this estimate were wide (0.65–5.06). The other genotypes (6A/8A, 7A/9A, 8A/9A and 9A/10A) were combined to determine whether they affected ovarian cancer risk. The frequency of rare alleles was somewhat higher in cases (1.7%) compared to controls (1.14%), but this difference was not significant (OR = 1.71, 95% CI 0.62–4.70). None of the above results were appreciably different when Caucasian and African-American subjects were analyzed separately and when analyses were performed after excluding the 134 borderline tumors.

The relationship between the *TGFβR1* polymorphism and risk of various histologic types of ovarian cancer is demonstrated in Table 3. Those with serous histology comprise 60% of the cases and the odds ratios in this subset were similar to those seen in the entire group. In the other histologic types, there were too few 6A homozygotes to allow calculation of odds ratios and these were combined

Table 2
Relationship between *TGFβR1* polymorphism and ovarian cancer risk in Caucasian and African-American subjects

Genotype	All	Races					Caucasians						African-Americans					
	Controls				Cases		Controls			Cases			Controls			Cases		
	n	(%)	*n*	(%)	OR[a]	95% CI	*n*	(%)	*n*	(%)	OR[b]	95% CI	*n*	(%)	*n*	(%)	OR[b]	95% CI
9A/9A	497	(81%)	468	(80%)	1.00	reference	415	(80%)	390	(79%)	1.00	reference	73	(88%)	63	(82%)	1.00	reference
6A/6A	6	(1%)	10	(2%)	1.81	(0.65–5.06)	6	(1%)	10	(2%)	1.89	(0.68–5.29)	0	(0%)	0	(0%)	Too few to calculate	
6A/9A	104	(17%)	100	(17%)	1.03	(0.76–1.40)	98	(19%)	93	(19%)	1.02	(0.74–1.40)	4	(5%)	6	(8%)	2.05	(0.54–7.87)
6A/6A or 6A/9A	110	(18%)	110	(19%)	1.07	(0.80–1.44)	104	(20%)	103	(21%)	1.07	(0.79–1.45)	4	(5%)	6	(8%)	2.05	(0.54–7.87)
Other	7	(1%)	10	(2%)	1.71	(0.62–4.70)	1	(0%)	2	(0%)	Too few to calculate		6	(7%)	8	(10%)	1.81	(0.57–5.74

[a] Age and race adjusted.
[b] Age adjusted.

Table 3
Relationship between *TGFβRI* polymorphism and risk of histologic subtypes of ovarian cancer

Genotype	Controls		Serous				Endometrioid/clear cell				Mucinous			
	n	(%)	*n*	(%)	OR[a]	95% CI	*n*	(%)	OR[a]	95% CI	*n*	(%)	OR[a]	95% CI
9A/9A	497	(81%)	277	(78%)	1.00	reference	88	(81%)	1.00	reference	54	(77%)	1.00	reference
6A/6A	6	(1%)	6	(2%)	1.92	(0.61–6.08)	1	(1%)	Too few to calculate		1	(1%)	Too few to calculate	
6A/9A	104	(17%)	61	(17%)	1.06	(0.75–1.51)	18	(17%)	0.98	(0.56–1.71)	15	(21%)	1.32	(0.70–2.49)
6A/6A or 6A/9A	110	(18%)	67	(19%)	1.11	(0.79–1.56)	19	(18%)	0.97	(0.56–1.68)	16	(23%)	1.32	(0.71–2.45)
Other	7	(1%)	9	(3%)	2.45	(0.86–6.93)	1	(1%)	Too few to calculate		0	(0%)	Too few to calculate	

[a] Age and race adjusted.

with 6A/9A heterozygotes. There was no suggestion of an association with endometrioid or clear cell cancers. Although there was a small increased risk of the mucinous subtype (OR = 1.32, 95% CI 0.71–2.45), the confidence intervals were wide and include one. Finally, there was no relationship between the polyalanine polymorphism and stage (I/II vs. III/IV) or tumor behavior (borderline vs. invasive) or stage (Table 4).

Discussion

The polyalanine repeat polymorphism in the type I TGFβ receptor was identified in 1998 in the context of mapping the gene to chromosome 9q22 [12]. A variant allele was described with an inframe deletion of 3 alanine residues from a nine residue stretch. Although cells with the 6A variant were shown to retain sensitivity to the growth inhibitory effects of TGFβ, an increased frequency of the 6A allele was noted in a group of patients with cancer compared to controls. In the ensuing years, several case-control studies have been performed to determine whether the *TGFβR1* 6A allele predisposes to various types of cancers. A recent meta-analysis that pooled data from various studies concluded that the 6A allele increases risk of breast, ovarian and colorectal cancer [10]. However, most of the reported studies suggesting an association with cancer risk have not been comprised of carefully matched cases and controls. Population admixture and other potential confounders in such studies may lead to false-positive associations. In this regard, Lai pointed out that a meta-analysis of the various studies is problematic because of differences in patient characteristics, the way in which cases and controls were sampled and the types of cancers studied [13]. Formal analysis demonstrated a lack of homogeneity in the studies, which would preclude pooling the data.

Prior studies of the *TGFβR1* polymorphism in ovarian cancer have been inconsistent. The 6A allele was first associated with ovarian cancer susceptibility in the context of a study that predominantly focused on colon cancer risk [8,9]. This study included only 48 ovarian cancer cases and 8 (17%) carried the 6A allele, compared to 12% of controls. Baxter et al. examined the relationship between the polyalanine polymorphism and ovarian cancer risk in 304 cases and 248 controls from the United Kingdom [9]. All subjects were Caucasian and 16.5% of controls were found to carry one or two copies of the 6A allele. There was no overall association of the 6A allele with ovarian cancer susceptibility. However, subgroup analysis revealed an increased risk of endometrioid and clear cell ovarian cancers (OR = 2.1, 95% CI 1.2–3.6). The control group in this study was comprised of staff volunteers and patients attending an obstetric clinic; and controls had a mean age of 39 compared to 62 in cases. The considerably younger age among controls in this study compared to cases is not ideal and could contribute to spurious results as endometrioid/clear cell cancer ovarian cancers and endometriosis both increase with aging.

The most recent meta-analysis of the *TGFβR1* polyalanine polymorphism by Pasche included 409 ovarian cancers [14]. There were 304 cases from the United Kingdom study and an additional 105 hospital-based ovarian cancers from the United States. Those either heterozygous or homozygous for the 6A allele had an increased risk of ovarian cancer (OR = 1.4, 95% CI 1.02–1.95) when compared to the large group of controls that were pooled in the meta-analysis. Based on less than 10 ovarian cancer cases who were 6A homozygotes, it was also concluded that the risk of ovarian cancer was more pronounced in this group (OR = 2.69, 95% CI 1.08–6.71).

The North Carolina Ovarian Cancer study is a population-based case-control study that is being conducted in the eastern and central areas of the state. Ovarian cancer cases are age and race matched to controls. In addition, controls must be at risk for ovarian cancer by virtue of having at least one ovary. Additional strengths include rapid case ascertainment, central pathology review and the availability of data regarding risk factors known to affect ovarian cancer susceptibility. The study population has also been previously analyzed for other polymorphisms including those in BRCA1 and 2 and the progesterone receptor [15,16]. A polymorphism in the promoter of the progesterone receptor was found to be associated with increased risk of endometrioid/clear cell ovarian cancers, and this finding was confirmed in a second case-control study conducted by collaborators in Australia [17].

Because a significant fraction of subjects in the North Carolina ovarian cancer study are African-American, we were able to examine racial differences in allele frequencies

Table 4
Relationship between TGFβ-RI polymorphism and risk of ovarian cancer by stage and behavior

All races			Tumor stage						Tumor behavior					
	Controls		I/II			III/IV			Borderline			Invasive		
Allele	*n*	(%)	*n*	(%)	OR[a]	95% CI	*n*	(%)	OR[a]	95% CI	*n*	(%)	OR[a]	95% CI	*n*	(%)	OR[a]	95% CI
9A/9A	497	(81%)	206	(82%)	1.00	reference	256	(78%)	1.00	reference	108	(82%)	1.00	reference	359	(79%)	1.00	reference
6A/6A	6	(1%)	4	(2%)	1.39	(0.38–5.06)	5	(2%)	1.96	(0.58–6.60)	3	(2%)	1.68	(0.40–7.15)	7	(2%)	1.78	(0.59–5.40)
6A/9A	104	(17%)	40	(16%)	0.89	(0.60–1.34)	58	(18%)	1.13	(0.79–1.62)	17	(13%)	0.76	(0.43–1.34)	83	(18%)	1.12	(0.81–1.54)
6A/6A or 6A/9A	110	(18%)	44	(18%)	0.92	(0.62–1.37)	63	(19%)	1.17	(0.82–1.67)	20	(15%)	0.82	(0.48–1.41)	90	(20%)	1.15	(0.84–1.58)
Other	7	(1%)	0	(0%)	Too few to calculate		10	(3%)	2.71	(0.97–7.59)	4	(3%)	2.94	(0.74–11.69)	6	(1%)	1.26	(0.40–3.97)

9 missing stage.
[a] Age and race adjusted.

of the *TGFβR1* polyalanine polymorphism. The frequency of the 6A allele was significantly lower in African-Americans (2.4%) compared to Caucasians (10.7%). This provides the first clear evidence that population admixture is a critical factor in case-control studies of this polymorphism and raises concern regarding prior studies that employed heterogeneous groups of subjects without carefully controlling for race [10]. The data presented in this paper represent the largest and most epidemiologically rigorous study to examine the relationship between the *TGFβR1* polyalanine polymorphism and ovarian cancer risk. Overall, we found that 18% of controls were carriers of the 6A allele compared to 17% in the meta-analysis reported by Pasche. There was no association between the 6A allele and ovarian cancer risk in the North Carolina Ovarian Cancer study (OR = 1.07, 95% CI 0.80–1.44). In addition, in contrast to the British study, we did not find an association between the *TGFβR1* 6A allele and clear cell/endometrioid histologic subtypes [9]. The inconsistency of these results suggests that the original finding was a false positive association. The *TGFβR1* 6A was also non-significant for an increased risk of serous or mucinous cancers. Likewise, there was no association specifically with stage or tumor behavior (borderline vs. invasive).

A population-based study of colon cancer in Utah also failed to confirm the association between the *TGFβR1* 6A allele and colon cancer susceptibility [11]. Likewise, other studies in colon cancer [18] and bladder cancer [19] did not find that the 6A allele increased risk. These studies and the present report suggest that heterozygosity for the 6A allele does not increase cancer risk. Because of the rarity of 6A homozygotes, none of the studies performed to date has had adequate power to determine with certainty whether this genotype increases risk. In the present study, cases were almost twice as likely to be 6A homozygotes compared to controls, but the rarity of this genotype (1.7% versus 0.98%) precludes a definitive conclusion. Larger studies would be needed to address this issue, as well as the effect of rare alleles (7A, 8A, 10A) in African-Americans. Even if it were shown that 6A homozygotes or those with other rare alleles are at increased risk of ovarian and other cancers, the clinical implications would not be great as very few individuals carry these genotypes.

Acknowledgments

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