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Association between telomere length and chromosome 21 nondisjunction in the oocyte

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Abstract

Chromosome 21 nondisjunction in oocytes is the most common cause of trisomy 21, the primary chromosomal abnormality responsible for Down syndrome (DS). This specific type of error is estimated to account for over 90% of live births with DS, with maternal age being the best known risk factor for chromosome 21 nondisjunction.

The loss of telomere length and the concomitant shortening of chromosomes is considered a biological marker for aging. Thus, we tested the hypothesis that mothers who had a maternal nondisjunction error leading to a live birth with DS (N=404) have shorter telomeres than mothers with live births without DS (N=42). In effect, our hypothesis suggests that mothers of children with DS will appear “biologically older” as compared to the mothers of euploid children. We applied a quantitative PCR assay to measure the genome-wide relative telomere length in order to test this hypothesis.

The results of our study support the hypothesis that young mothers of DS babies are “biologically older” than mothers of euploid babies in the same age group and supports telomere length as a biomarker of age and hence risk for chromosome nondisjunction.

Keywords

telomere length; nondisjunction; chromosome 21; Down syndrome

Introduction

Trisomy 21, the common chromosomal abnormality leading to Down syndrome (DS), has become an important model for human chromosome nondisjunction. At least 90% of chromosome 21 nondisjunction errors occur in the oocyte (Freeman et al. 2007), this percentage depending primarily on the structure of maternal age at the time of conception in the sample being studied. The strong association between advanced maternal age and nondisjunction of chromosomes 21 is found for errors that are classified as maternal meiosis I (MI) and as maternal meiosis II (MII). Thus, the impact of maternal age plays a critical role throughout the development of the oocyte. To review this timeframe, the initial step of oogenesis begins early in the fetal life of the female where meiosis is initiated. MI is arrested

in prophase after chromosomes synapse and recombine. It then resumes in the woman's adult life when the oocyte is ovulated. At this point, MI is completed and the first polar body is extruded. MII begins, but arrests for a short period as the oocyte travels down the fallopian tubes. MII is completed after fertilization and the second polar body is extruded. Thus, meiosis in a woman extends over a 10 to 50 year period and by extension, the age of the woman at conception reflects that age of the oocyte, and primarily the period of arrest in MI.

There is growing evidence that risk factors associated with MI and MII nondisjunction errors differ (Sherman et al. 2007), not unexpected given the mechanistic differences and temporal separation of MI and MII. Two dominant risk factors, maternal age and recombination profile along the nondisjoined chromosomes 21, show different patterns among maternal MI and MII errors (Allen et al. 2009; Lamb et al. 2005; Lamb et al. 1996). For example, mothers of infants with trisomy 21 due to a MII error are older at the time of birth compared with those due to MI error (Allen et al. 2009). With respect to altered recombination patterns along the nondisjoined chromosome 21, single telomeric recombinant events increase the risk for MI errors while pericentromeric recombinant events increase the risk for MII errors (Lamb et al. 2005; Lamb et al. 1996; Oliver et al. 2008; Oliver et al. 2012).

Since replicative shortening of telomeres can be considered a marker of biological aging (Aviv 2008; Martinez et al. 2009), it may also serve as a marker for the biological age of the ovary or perhaps a marker for environmental exposures. Many hypotheses for the maternal age effect on nondisjunction are based on the degradation of the meiotic components in the aged ovary (Yoon et al. 1996). If true, we may expect to observe shorter telomeres among mothers with nondisjunction events compared to mothers without observed nondisjunction, adjusting for chronological age. Based on this prediction, Ghosh et al. (2010) compared the telomere lengths measured in peripheral blood lymphocytes of mothers of trisomic offspring (case mothers=75) to mothers with euploid offspring (control mothers=75). They stratified cases by MI (n=48) and MII errors (n=27) and performed linear regression to compare telomere length as a function of age in the three meiotic outcome groups. They showed that all three groups had similar telomere lengths on average for young mothers (18-25 years old) and middle age mothers (26-34 years old) and all three groups declined with age. However, for the older age group (35-42 years old), telomere length was shortest in the MII case group and longest in the control group, with the MI case group in between. These results do not support the hypothesis that young mothers with nondisjunction events have biologically older ovaries than their chronological age. However, the shorter telomeres lengths in later years may be a surrogate for increase age-related exposures that affect the ovarian environment.

Here, we further test the hypothesis of the premature "biological aging" among mothers who had a maternal nondisjunction error leading to a livebirth with DS (case mothers=404) compared with mothers with livebirths without DS (control mothers=42) using shortened telomere length as an indicator. Telomere length was measured as the genome-wide relative telomere length (T/S ratio) according to a modified protocol of the method described by Cawthon et al. (2002). Analyses were stratified by type of meiotic error. The results of our

study support the hypothesis of the premature “biological aging” model for younger mothers (Warburton 2005). Based on the T/S ratios, the results suggested that young mothers (<35 years old) of DS babies are “biologically older” than mothers of euploid babies in the same age group. Based on these results, telomere length could be considered a biomarker of age and hence nondisjunction.

This study provides new insight into the relationship between maternal age, telomere length and the nondisjunction of chromosome 21 and could be the basis of future studies leading to a better understanding of the maternal age risk factor leading to chromosome 21 nondisjunction.

Subjects and Methods

Ethics Statement

This work was approved by the Emory University Institutional Review Board (IRB) and by the local IRB of the institution at each recruitment site.

Sample Description

Cases were defined as mothers who had an infant with trisomy 21 due to a maternal nondisjunction error (n=404). Controls were defined as mothers who had an infant with trisomy 21 due to a paternal error or a post-zygotic mitotic error (n=24) or mothers drawn randomly from the general population who had an infant without trisomy 21 (n=18). For both cases and controls, their blood sample was required to have been drawn within 5 years of the birth of the index case. DNA extraction was performed following the protocol from Gentra Systems, Puregene Genomic DNA purification kit from venous blood samples. After genomic DNA was extracted, samples were quantified and then stored at 4°C.

We genotyped over 1500 chromosome 21-specific polymorphisms that span 21q in the proband with DS, mother and father to characterize the type of nondisjunction error. A detailed description of the markers, genotyping quality control measures, and method is provided in Oliver et al. (2012).

Determination of Type of Nondisjunction Error

Using the genotype data from the proband and their parents, we first defined parental origin of the meiotic error by determining the contribution of parental alleles to the proband with trisomy 21. At least two markers had to provide consistent information to establish parent origin. Once defined, we determined the type of meiotic error that gave rise to the trisomy. In other words, we determined whether the error happened during the meiosis I (MI) or the meiosis II (MII). We used polymorphisms located in the pericentromeric region of chromosome 21q, more specifically between base locations 13,615,252 and 16,784,299. If parental heterozygosity was maintained in the proband at a pericentromeric marker, we concluded that the nondisjunction error happened during MI. If parental heterozygosity was reduced to homozygosity in the proband, we concluded that the error happened during the MII. One of the caveats of our method is that this assay cannot distinguish between the different types of underlying errors that might lead to an MII error (Oliver et al. 2012; Oliver

et al. 2008). Lastly, we defined a post-zygotic mitotic error when all informative markers of the parent of origin along 21q were reduced to homozygosity in the proband.

Determination of Telomere Length

Relative telomere length was measured according to a modified protocol of the method described by Cawthon et al. (2002). This method using quantitative PCR proved to be rapid and more importantly, allowed us to obtain reproducible estimates of relative telomere length with the use of only 30ng of genomic DNA. Briefly, two master mixes of PCR reagents were prepared, one with the telomere (T) primer pair and the other with the single copy gene (S) primer pair. Similar to Cawthon et al. (2002), we used 36B4 gene as a single copy gene, which encodes acidic ribosomal phosphoprotein PO and is located on chromosome 12 (Boulay et al. 1999). The final concentrations of reagents in the PCR were 0.16× Sybr Green I, 15 mM Tris-HCl pH 8.0, 50 mM KCl, 1.5 mM MgCl₂, 0.16 mM each dNTP, 5 mM DTT, 1% DMSO and 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems).

The final telomere primer concentrations were: tel 1, 216 nM; tel 2, 720 nM. The final 36B4 (single copy gene) primer concentrations were: 36B4u, 240 nM; 36B4d, 400 nM. The primer sequences were as follows:

TEL 1: 5' – GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT – 3'

TEL 2: 5' - TCCCGACTATCCCTATCCCTATCCCTATCCCTATCC-CTA – 3'

36B4u: 5' – CAGCAAGTGGGAAGGTGTAATCC – 3'

36B4d: 5' – CCCATTCTATCATCAACGGGTACAA – 3'

All PCRs were performed on the Bio-Rad CFX96 real-time PCR detection system. The thermal cycling profile for both PCRs began with a 95°C incubation for 10 min to activate the AmpliTaq Gold DNA polymerase. For telomere PCR, the protocol used consisted of 26 cycles of 95°C for 15 s, 54°C for 2 min. For 36B4 PCR, the protocol was 32 cycles of 95°C for 15 s, 58°C for 1 min. BIORAD software was used to generate the standard curve for each plate and to determine the dilution factors of standards corresponding to the T and S amounts in each sample.

Two PCR reactions were performed for each experimental sample: one to measure the single copy gene relative quantity and the another to measure the relative quantity of telomere repeats. A randomly chosen sample (reference sample) was used to generate a standard curve to which the results of the rest of the samples (cases) were compared.

The ratio of these two PCR results for each experimental sample was defined as the relative telomere to single copy gene (T/S) ratio. We conducted two replicates on each sample to control for experimental variation: two identical aliquots of the DNA sample were added to plate 1 to assay telomere repeat copy and another two aliquots were added to the same well positions in plate 2 to assay the single copy gene. For each standard curve, one reference DNA sample was diluted serially to produce five total amounts of DNA: 20 ng, 10 ng, 5 ng, 2.5 ng and 1.25 ng.

For quality control, the efficiency of the PCR reaction and the r-square value of the regression line created by the standard dilutions were examined for each reaction. The efficiency of the PCR reaction measured by the threshold cycles of the standard dilutions used to create the regression line had to be between 90% and 110% and the r-square value of the regression line had to be no less than 0.98. If any of these parameters were not met, the entire PCR for the plate was repeated. We also applied a quality control to each of the duplicates run for each sample. The duplicate values for each sample could not have a threshold cycle value of more than 0.3 cycles apart. If this parameter was not met, both the T PCR and the S PCR for that sample were repeated.

Statistical Analysis

We examined T/S ratios by maternal age using linear regression models, stratified by meiotic outcome group (MI errors, MII errors and controls). Table I shows the different sample sizes for each analyses used in the different linear regression models. We then included an interaction term between maternal age and meiotic outcome group (MI vs controls, MII vs controls, and MI vs MII) in the regression models to test whether the pattern of maternal age on T/S ratio across the age span differed by meiotic outcome group. To be comparable to the study of Ghosh et al. (2010), we also conducted t-tests between maternal age groups, stratified by meiotic outcome groups. We also included race/ethnicity in our models and found that it was not significant ($p>0.20$, data not shown); thus, it was excluded from the final regression models.

Results

Using the study sample outlined in Table II, we first sought to confirm the negative relationship between telomere length and maternal age. Indeed our results indicated a statistically significant association of T/S ratio measured by quantitative PCR and maternal age in both controls ($p=0.013$, Table I, Fig. 1) and all maternally-derived nondisjunction cases ($p<0.0001$, Table I). As our further analyses required maternally-derived nondisjunction cases to be defined by stage of origin, we conducted the above analysis on the subset of cases that were informative for stage of origin ($N=241$) to confirm the association of maternal age on T/S ratio. In this select group, we found the same statistically significant association between T/S ratio and maternal age ($p=0.009$, Table I, Fig. 2). This suggests the selected cases on which we conducted further analyses were representative of the overall group of maternal errors.

In our next set of analyses, we stratified maternally-derived errors based on the stage of the error, namely MI or MII. We first examined the mean T/S ratio by maternal age group, as defined in Ghosh et al. (2010), to provide a comparison of the pattern of the mean telomere length by age for the two studies (Table III). We note that a direct comparison cannot be made, as each study used a different measure of telomere length. Irrespective, differences between studies were observed. First to review, Ghosh et al. (2010) did not observe a difference in mean telomere length among young and middle age women from the different meiotic outcome groups, but did in the older groups. In our analyses, we collapsed the two younger age groups due to small sample sizes and compared the means of women less than

35 years old. In this younger group (<35 yrs), we found that T/S ratio among controls (mean=1.90, SD=0.78) was significantly greater than MI error cases (mean=1.61, SD=0.39, $p<0.01$) and greater than MII error cases (mean=1.51, SD=0.4, $p=0.02$). There was no difference in mean T/S ratio between MI and MII error groups ($p=0.26$). In the older group (>35 yrs), we found no difference in the mean T/S ratios among controls (mean=1.44, SD=0.21) vs MI (mean=1.55, SD=0.51, $p=0.41$) and controls vs MII (mean=1.49, SD=0.30, $p=0.57$), nor between MI vs MII error groups ($p=0.41$).

We then conducted regression analysis to examine the effect of maternal age on telomere length over the entire maternal age range (continuous variable), stratified by meiotic outcome group. We found that there was a significant decrease in T/S ratio by maternal age among controls and MI errors, but not MII errors (Fig. 1, 3, 4, respectively; Table I). To determine whether the magnitude of the decrease in T/S ratio by maternal age differed among the meiotic outcome groups, we conducted a joint regression analysis that included an interaction term for maternal age by meiotic outcome group for the following datasets: MI and controls, MII and controls, and both MI and MII. For both MI and MII errors, the influence of maternal age on the T/S ratio differed significantly from controls, as indicated by a statistically significant beta coefficients for the interaction terms ($p=0.03$ and $p=0.02$, respectively). The interaction term was not statistically significant when MI and MII errors were examined together ($p=0.39$).

Sensitivity analysis

Our T/S ratios measurements showed four values that were greater than two standard deviation above the mean value: two were from MI error cases and two from controls. We repeated the quantitative PCR analysis in all samples, but obtained the same results for these four outliers. We had no reason to remove these samples from the analysis, but we wanted to address how much these outliers influenced the results. We conducted the same regression analyses with these four samples excluded. All results were similar with the exception of the comparison between controls and MI errors. When the outliers were removed in the joint analysis of controls and MI errors, the significance for the maternal age-meiotic outcome group interaction terms was reduced from 0.03 to 0.10.

Discussion

The effect of advanced maternal age on the risk of having a child with DS was established nearly 80 years ago (Penrose 1933). This association is now known to be due to the attributes of the aged oocyte and its meiotic machinery (Morton et al 1988; Risch et al. 1986).

Several studies in mice (Liu et al. 2004; Liu et al. 2002) and in women (Keefe et al. 2007; Scherthan et al. 1996; Scherthan 2006; Roig et al. 2004) have shown an association between telomeres and the meiotic machinery. These studies showed the importance of functional telomeres for the correct chromosome segregation during meiosis. Also, as replicative shortening of telomeres is considered a marker of biological aging (Aviv 2008; Martinez et al. 2009), it may also serve as a marker for the biological age of the ovary or perhaps a marker for environmental exposures. Thus there are two possible explanations that could

explain the association between telomere length and age-related chromosome nondisjunction. One explanation may be that shortened telomeres that result from biological aging do not function as well as longer telomeres while the other explanation may be that shortened telomeres are simply a marker of aging and point to accelerated aging in women who have had nondisjunction events.

In 2010, Ghosh et al. (2010) reported the possible relationship between telomere length in mothers who had a child with DS and the risk of nondisjunction. They compared a group of 75 mothers of euploid children to a group of 75 mothers of children with DS. From this last group, 48 were known to have maternal MI nondisjunction errors and 27 with maternal MII errors. They found that young and middle age women among the different meiotic outcome groups did not differ with respect to their telomere length whereas those in the older age group did: both MI and MII case mothers had shorter telomeres compared with controls, especially MII case mothers. They concluded that these results did not support the hypothesis that younger women who have babies with DS are 'biologically older' than their chronological age. Instead, their findings suggested that older mothers with a nondisjunction error looked "biologically" older than controls, perhaps having more rapid telomere loss due to genetic or environmental factors.

Our study took advantage of a larger sample size of mothers who had children with MI and MII errors in order to confirm or refute the association between telomere length, maternal age and the risk of nondisjunction of chromosome 21. Our first goal was to confirm a negative relationship between telomere length and age, as pointed out by previous studies (Takubo et al. 2002; Hoffmann et al. 2009; Ishii et al. 2006). Indeed, our results indicate a statistically significant association of T/S ratio measured by quantitative PCR and maternal age in both controls (N=42) and all maternally-derived nondisjunction cases (N=404) (Table I). Our results suggest that controls show longer telomere length in younger women compared to younger women with maternally-derived errors. These results support the hypothesis that oocytes with a nondisjoined chromosome 21 may represent a biologically older ovary compared with those resulting in a meiotically normal outcome. Previous studies in mice would support this hypothesis (Liu et al. 2004; Liu et al. 2002; Liu and Keefe 2002).

We further examined the pattern of the association stratified by MI and MII error groups. There was a significant association between telomere length and age in the MI error group, but we did not observe it among the MII error group (Table I, Fig. 4). The MII error group shows the shortest telomere length in young women compared with controls and MI errors (Tables I, III), although differences between MI and MII were not statistically significant. This result suggests that MII mothers are "biologically" older than women of the same age in the control or MI groups and that they could represent a group of women who are at high risk for nondisjunction because of their shortened telomeres. Although the sample size is small for the MII error group, we did not observe the significant decrease in telomere length by age as did Ghosh et al. (2010).

We have considered several possible explanations for the different findings between our study and that of Ghosh et al. First of all, our sample sizes for MI and MII cases were larger than that in the Ghosh et al. study (48 vs 190 MI cases, 27 vs 51 MII cases), although our

sample of controls was smaller (42 vs 75). While our control sample size was smaller, it did show a statistically significant decline in telomere length with age as expected. Nevertheless, some differences in findings may be due to limited sample sizes. Another technical issue may be the use of different assays to assess telomere length, although both technologies have been shown to provide comparable results (Cawthon 2002). Lastly, many studies suggest that telomere length is affected by environmental exposures (Epel 2009; Entringer et al. 2013). Thus, the different observed age-related patterns in telomere length may be explained by differences in the environment among women representing the U.S. and those from Kolkata, India.

Assuming that our results are representative of the women in the different meiotic outcome groups collected within the U.S., we summarize our findings and suggest the following interpretations. First, our data show that younger women with MI and MII errors (<35 yrs) had significantly shorter telomeres than controls. These data suggest that younger women with nondisjunction errors are “biologically” older than controls, especially for those with MII errors. Thus, telomere length may serve as a biomarker of risk for nondisjunction in this chronologically younger age group.

As maternal age increased, the difference in telomere length among the meiotic outcome groups became smaller. Of the two types of errors, the MII mothers, appeared to be the biologically “oldest” group, with a T/S ratio significantly smaller at young ages compared to controls and with no evidence of a decrease with maternal age. We must be cautious in our speculation that women with MII errors may be the biologically “oldest” meiotic outcome group, as there was no statistically significant difference in the age patterns between the MI and MII groups.

Although our data are not longitudinal, only cross-sectional, we speculate about the suggested pattern of telomere length decline. Studies in humans have shown that telomeres do not decline in a linear way over the age span (Aubert and Lansdorp 2008): from the first years of life until around 30-35 years old, telomere attrition steadily declines; from 35 to about 60 years of age, telomere length attrition slows; and, after age 65, the decline is more rapid. A possible explanation for the reduced slope in the age-related pattern among mothers with nondisjoined events is that their telomeres may be in a biologically older stage, where the decline over time is reduced. That is, the attrition pattern of the telomeres of the younger mothers with nondisjunction in our dataset may be similar to that typical to mothers in an age range of 35-60 years of age.

We also noticed that the regression lines for maternal age on T/S ratio among the different meiotic outcome groups converge around 35-37 years of age. Interestingly, this is the age where the risk for nondisjunction of chromosome 21 changes from a linear increase to an exponential increase (Allen et al. 2009). One possible explanation for this pattern is that telomere length is an important biomarker of biological age and that biological age is strongly associated with nondisjunction risk. That is, the controls in our dataset reach their “35-37 year” telomere length at their chronological age because their biological age tracks closely with their chronological age. In contrast, mothers with nondisjunction error reach that high-risk “35-37 year” telomere length earlier due to their increased biological aging. If

these data are confirmed in a larger sample, it will be important to understand whether shortened telomere length is simply a biological marker of aging or actually is a risk factor for nondisjunction due to its involvement in chromosome segregation during meiosis.

Study Limitations

Although our study is the largest study to date on the influence of telomere length on the risk of maternal nondisjunction leading to DS, there are still some limitations that future studies could overcome. A larger sample size among control mothers with a wide range of ages at the time of blood draw would be advisable in order to better estimate the slope of the regression line of telomere length and maternal age. Similarly, a larger sample size of mothers with MII errors would be advisable to determine whether MI and MII error groups differ in their pattern of age-associated telomere length. This may be difficult to achieve, as only 25% of maternal errors correspond to this group (Freeman et al. 2007; Sherman et al. 2007; Gomez et al. 2000). Irrespective, we are currently trying to obtain additional samples and genotyping on the 163 maternal cases where we were unable to detect the stage of the meiotic error. A larger sample size would improve our power to observe possible differences and to confirm our results. Lastly, as stated above, our data were cross-sectional and thus our interpretations about the decline in telomere length by age are only speculative. A better study design would be to sample women at several different time points in their lifetime to study true loss of telomere length. This may require a prospective study of women in their reproductive stage to obtain estimates of telomere length from a young age to beyond the age of the nondisjunction event. These data would be important to determine whether telomere length measured at a young age is predictive of risk for nondisjunction and thus serve as an important biomarker.

Conclusions

Our results support the hypothesis of the premature “biological aging” model for younger mothers (Warburton 2005). Based on our T/S ratios measurements, we propose that young mothers with a nondisjunction event leading to DS are “biologically older” than mothers of euploid live births in the same age group. Based on these results, telomere length could be considered a biomarker of biological age and hence of risk for nondisjunction. This study provides new insight into the relationship between maternal age, telomere length and the nondisjunction of chromosome 21 and serves as a basis of future studies leading to a better understanding of the maternal age risk factor leading to chromosome 21 nondisjunction. Future studies that focus on longitudinal measures of telomere length over the reproductive age span of a woman, along with assessments of important environmental exposures could provide additional insight to this important association.

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Fig. 1. Regression line and observed data points showing the distribution of T/S ratio as function of maternal age among control mothers.

Fig. 2. Regression line and observed data points showing the distribution of T/S ratio as function of maternal age among case mothers.

Fig. 3. Regression line and observed data points showing the distribution of T/S ratio as function of maternal age among MI error mothers.

Fig. 4. Regression line and observed data points showing the distribution of T/S ratio as function of maternal age among MII error mothers.

Table I

Results of the linear regression models predicting T/S ratio using maternal age at blood draw for controls for all maternally-derived nondisjunction (Mat NDJ) cases, those informative for stage of origin, and for MI and MH errors separately.

Meiotic outcome group	Sample size	Intercept (SE)	β coefficient for maternal age (SE)	p-value for maternal age	R ² for model
Controls	42	3.170(0.555)	−0.044(0.016)	0.013	0.146
Mat NDJ: all women	404	2.027(0.097)	−0.013(0.002)	<0.0001	0.054
Mat NDJ: Subset with type of error identified	241	1.959(0.151)	−0.012(0.004)	0.009	0.028
MI errors	190	2.025(0.173)	−0.013(0.005)	0.011	0.034
MII errors	51	1.619(0.299)	−0.003(0.008)	0.689	0.003

(SE: standard error)

Table II

Mean age at time of blood draw of mothers and sample size stratified by age group and by meiotic outcome groups: controls, MI errors and MII errors.

Maternal age group	Mean age at Mood draw (sample size)		
	Controls	MI errors	MI errors
18-34 years	29.2 (n=27)	28.7 (n=100)	29.9 (n=27)
35 yrs	39.3 (n=15)	38.7 (n=90)	39.4 (n=24)

Table III

Mean T/S ratio and standard deviation (SD) by meiotic outcome group stratified by maternal age group at blood draw.

	Mean age at Mood draw (sample size)		
Maternal age group	Controls	MI	MII
18-34 years	1.90 (0.78)	1.61(0.39)	1.51(0.4)
35 years	1.44(0.21)	1.55(0.51)	1.49(0.30)