STUDIES OF ASSOCIATION OF ENVIRONMENTAL RISK FACTORS IN DOWN SYNDROME

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Down syndrome (DS) is the most common live-born birth defect in humans. The genetic cause of DS is trisomy of chromosome 21. 94% of cases of DS trisomy are due to maternal chromosomal non-disjunction. Advanced maternal age has been identified as a key risk factor for DS. However, it is not clearly understood yet how maternal age effect increases chromosome non-disjunction. Recently, it has been suggested that factors involved in meiotic chromosome segregation may be altered in aged oocytes and such molecular abnormality could be caused or accelerated by maternal environmental factors. Epidemiologic studies have tried to investigate effect of maternal environmental factors on DS. The most commonly-studied factors are maternal peri-conceptional smoking and oral contraceptive use. However, the evidence of association of those factors with DS has not been consistent. In this thesis, a DS cohort in Kolkata, India was investigated for studying association of maternal peri-conceptional behavioral factors on DS. The characteristics of exposure to two factors, smokeless chewing tobacco use (SCT) and oral contraceptive use (OC), are unique in this cohort as compared to previous study populations. In this population both exposures tend to be started early in life and in high dose. By using logistic and linear regression methods, I found significant association of maternal SCT and OC use with DS. SCT interacts with maternal age, having a stronger effect in younger mothers compared to older mothers, while the effect of OC use on risk is the same at all ages. SCT use is also associated with meiotic type (I vs. II) of chromosome non-disjunction and with lack of recombination in meiosis I type non-disjunction. Furthermore, maternal SCT use is strongly associated with maternal telomere length, which has been shown to be a molecular marker of aging. SCT use decreases maternal telomere length, especially among women bearing DS child with meiosis I type non-disjunction. In conclusion, with Indian DS cohort, this thesis was able to find evidence of interactions among three major risk factors in DS: tobacco use, oral contraceptive use, and maternal age. This study has great public health significance because better understanding of the risk factors for DS can lead to improved prevention and screening strategies.

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PREFACE

I would like to thank my thesis advisor, Dr. Eleanor Feingold for giving me a good opportunity for analyzing these interesting studies and for guiding me to the right direction. I am truly grateful to my husband, Dr. Jong-Hyeon Jeong for all his support. He taught me a lot with patience and took care of our kids while I was studying. Super-thanks to Emily and Michael who could understand that mommy had to do lots of homework!

1.0 INTRODUCTION

Down syndrome (DS) is the most common live-born birth defect in humans. The incidence of DS is about 1 birth per 800 to 1000 live births in United States. About 6000 new DS children are born each year (reviewed in Sherman et al. 2007).

The DS child has distinctive physical features including facial appearances of small chin, round face and almond-shaped eyes, shorter limbs, and poor muscle tone. Most DS children have mental retardation in the mild to moderate range, and delayed development of motor skills. In addition, DS individuals have higher risks of several diseases including congenital heart defects and Alzheimer's disease in their later life. Early childhood intervention including screening for common problems and also medical treatment has resulted in improvement of overall development of DS children and quality of life (reviewed in Roizen and Patterson, 2003).

The genetic cause of DS is trisomy of chromosome 21 either in whole or part, in which a nucleus carries an extra chromosome 21 in addition to normal pair of chromosomes 21. 95% of individuals DS carry standard trisomy of whole chromosome 21 and less than 5% carry somatic mosaicism or translocation of part of chromosome 21. The extent of the extra copy affects phenotypes of DS as well as severity of the defect. Full trisomy of chromosome 21 is usually caused by non-disjunction of meiosis during either oogenesis or spermatogenesis, although it can also be caused by mitotic error. 94% cases of DS trisomy are due to maternal non-disjunction while about 4% are due to paternal non-disjunction. Thus maternal factors appear as a major cause of DS (reviewed in Sherman et al. 2007).

Meiosis is a part of gamete formation in which the number of chromosomes reduces by half. Meiosis consists of two distinctive stages, I and II. Meiosis I in oocytes begins during female embryonic development. During this period homologous chromosomes pair and exchange segments resulting in

genetic recombination. This phenomenon is referred to as recombination. In this stage further process of meiosis I is arrested until puberty when meiosis I is resumed in each menstrual cycle and separation of homologous chromosomes (reduction division) is finished. Then proceeding in meiosis II is arrested until fertilization. This long period of arrest in oogenesis is presumed to increase risk of non-disjunction in oocytes compared to spermatogenesis in which meiosis occurs continuously without arrest (Suzuki et al. 1989).

Advanced maternal age as a key risk factor for DS was identified soon after DS began to be recognized epidemiologically in the mid 1800s. At maternal age 25, the probability of having a DS child is one in 1250 live births and at maternal age 35, the probability increases to one in 385. At age 40, it becomes one in 106 births (www.nichd.nih.gov, 2011). At this point, it is not clearly understood how the maternal age affects chromosome non-disjunction in oocytes, although it has been suggested that several factors involved in chromosome segregation such as adhesion and spindle molecules may be altered in aged oocytes (Zwick et al, 1999, Hawley, et al, 1994).

Recent molecular genotyping of chromosome 21 trisomy revealed that there are altered recombination patterns in DS. For example, studies along the maternal chromosome 21 in trisomic and euploid individuals have shown that absence of recombination is associated with increase of meiosis I type non-disjunction (MI NDJ), a single telomeric recombination is also associated with MI NDJ and a pericentromeric recombination is associated with meiosis II type non-disjunction (MII NDJ) (Ghosh et al., 2009, Lamb et al., 1996, 1997, 2005, Oliver et al., 2008).

Furthermore, there are numerous studies of molecular aging in cells. For example, the telomere length of chromosomes shortens with age. The telomere is the end structure of the chromosome, which consists of repetitive DNA sequence, $(TTAGGG)_n$ and is associated with proteins for protecting deterioration of chromosome after each cell division. It has been shown that there is significant correlation between shorter telomere length and cellular aging (Aviv, 2008). It has been speculated that

mothers of infants with DS may have advanced biological aging, and thus potentially shorter telomeres. However, a study did not find significance of telomere length between young mothers bearing DS child and age-matched controls (Dorland et al, 1998). Yet, since the telomeres shorten during DNA replication and also from the response to oxidative DNA damage which is accumulated in age (Keefe and Liu, 2009), this issue deserves further study. A recent study of Indian cohort has shown that telomeres shorten by function of ages and the loss is higher in cases than in control (Ghosh et al., 2010).

Although advanced maternal age is by far the strongest known risk factor, 80% of DS cases are born to mothers younger than age 35. Therefore it has been suggested that there must be multiple risk factors for DS (Hassold and Chiu, 1985). Several environmental risk factors for DS such as maternal smoking, drinking alcohol and oral contraceptive use at the time of pregnancy have been studied. For example, a population-based case-control study using Washington state birth record data showed no association between maternal smoking and risk of DS after controlling maternal age (odds ratio=1.0, 95% confidence interval 0.82-1.24) (Chen et al, 1999). Other studies showed negative association, suggesting that the maternal smoking reduces viability of DS conceptuses (Kline et al, 1993). More recent studies using molecular mapping of meiosis I and II error in DS cases show that periconceptional smoking increases meiosis II errors among mothers younger than 35 years (odds ratio=2.98, 95% confidence interval 1.01-8.87). The significance of odds ratio for DS appears to be augmented when smoking interacts with oral contraceptive use (odds ratio=7.62, 95% confidence interval=1.63-35.6) (Yang et al., 1999). Oral contraceptive use alone did not increase risk of DS in this study. Other environmental factors including maternal alcohol, irradiation and caffeine have not been shown to be significant for DS so far (Kaufman, 1983, Padmanabhan et al., 2004, Strigini et al., 1990). However because these studies were based on retrospective epidemiologic studies, it could include subjects' recall biases about their behaviors around the time of pregnancy, resulting in either over or underestimation of possible association of those risk factors with DS. Therefore, increasing the number

of subjects or studying a specific population that has been exposed densely with certain environmental factors may enhance the power to identify potential risk factors for DS. It can also be informative to compare risk factors in meiosis I and meiosis II error groups rather than comparing them to controls, as this eliminates the biases of a retrospective study.

1.1 OVERVIEW OF THESIS RESEARCH

This thesis is primarily focused on analyzing case-control study datasets to look for association of maternal environmental factors with chromosome 21 non-disjunction in Down syndrome. Two different Down syndrome datasets were received from Dr. Feingold's collaborator, Dr. Sujoy Ghosh at West Bangal University of Technology, India.

In Chapter 1, I describe analysis results of the first dataset which includes subjects of both cases and controls. The association study of maternal behaviors of smokeless chewing tobacco (SCT) use and oral contraceptive use (OC) with DS is described. I also describe case-only analyses for studying association of the maternal behaviors with MI or MII errors, recombination frequency and position.

In Chapter 2, I describe analysis results of the second dataset which contains only cases, overlaps with the first dataset in some cases and contains telomere measures. I found significant association of SCT use with maternal telomere length in DS cases.

2.0 ASSOCIATION OF MATERNAL PERICONCEPTIONAL BEHAVIORS IN CHROMOSOME 21 NON-DISJUNCTION OF INDIAN DOWN SYNDROME CASES

2.1 INTRODUCTION

Maternal periconceptional smoking or oral contraceptive uses on Down syndrome have been studied on several papers (for example, Chen et al., 1999, Kline et al, 1993, Yang et al., 1999). However, the results have been inconsistent, partly due to the study design or study population issues. To investigate association of these environmental factors on Down syndrome cases, this study used a very specific population in which subjects used smokeless chewing tobacco (SCT), a crude form of chopped tobacco leaves from early days of their adolescence and also used irregularly oral contraceptive pill even after they conceive. I studied interactions of the factors with maternal age, which is a key risk factor in Down syndrome, as well as with chromosome non-disjunction type and recombination frequency and position.

2.2 METHODS

2.1.1 Subjects

One hundred eighty three cases which are mothers with a live born DS infant were selected from Kolkata, India between 2002 and 2009. The research design was approved by the institutional ethics committee of the West Bengal University of Technology and an informed consent was taken from each subject. Eligibility criteria were availability of complete set of tissue samples from father, mother and DS infant and complete information of maternal life style, especially periconceptional SCT and OC use. DS infant has free trisomic chromosome 21 which was determined by classical karyotyping at Dr. Dey's laboratory. Interview of mothers was taken privately using a preprinted question set.

One hundred ninety five control mothers having a euploid infant were selected from the hospitals where cases were selected. Euploid infants were karyotyped as trisomic infants at the Dr. Dey's laboratory. Selection criteria for controls were kept on matching to cases in terms of ethnicity, language, religions and socio-economic status to reduce potential confounding factors between cases and controls (see Table 2.1). Eligibility for controls was also complete information of maternal habits and tissue samples of mother and child.

Table 2.1: Demographics of subjects

Characteristics	**Case** (n=183)	**Control** (n=195)
Mean maternal age (n)		
Young (≤28 years)	76 (41.53%)	59 (30.25%)
Middle (29-34 years)	59 (32.24%)	72 (36.92%)
Old (>34 years)	48 (26.23%)	64 (32.83%)
Locality		
Kolkata Metropolitan	105	110
Suburbs	71	79
Rural	8	5
Language speaking		
Bengali	167	173
Hindi	12	15
Others	4	8
Religions		
Hinduism	146	152
Islam	32	37
Others	5	6
Socio-economic Status		
High income (≥Rs.20,000)	22	25
Middle Income (Rs.5000-19,999)	95	110
Low income (≤ Rs. 5000)	66	60

2.1.2 Genotyping

A total of fifteen short tandem repeat (STR) markers covering the pericentromeric region to the telomere of the chromosome 21q arm were used for genotyping DS families. The parental origin of non-disjunction was determined by establishing contribution of parental alleles to the DS child. When at least three markers were informative and the allelic statuses of the rest of the markers were consistent, parental origin was assigned. Only maternal cases were included in the analyses presented here. Then MI NDJ or MII NDJ was determined. If the parental heteozygosity at the centromeric markers was retained in the DS child, it was considered as MI NDJ. If the parental heterozygosity was reduced to homozygosity at the DS child, then it was considered as MII NDJ. If MII NDJ had no recombination, then it was considered as a mitotic error and excluded from analyses.

The status of each marker in each DS child was recorded as non-reduced (N), reduced (R) or uninformative (U) and arranged from centromere to telomere of 21q. Recombination events were scored as of changes of status of two successive markers, such as R to N or N to R. When there were uninformative markers between intervals, the midpoint of the two intervals was given as the chiasma (recombination) position. For example, if the markers were NUR in the 2^{nd}, 3^{rd} and 4^{th} intervals, the chiasma position was scored as 2.5 and if the markers were NUUR in 2^{nd},3^{rd}, 4^{th} and 5^{th} intervals, the chiasma position was scored as 3.

The tissue samples from controls were also genotyped for eliminating mosaicism but those data were not included in the analyses.

2.1.3 Data management

Data from Dr. Ghosh were stored in Microsoft Excel files. Recorded variables for 183 DS cases are MI or MII NDJ, maternal age at conception, smokeless chewing tobacco (SCT) use, oral contraceptive (OC) use, and mapping of 15 STR markers. Using the STR map, recombination events and chiasma positions were scored in the Excel file. For 195 control mothers, maternal age at conception, SCT use and OC use were

recorded. Then the data was transferred into STATA software (version 9). Maternal age was categorized into three groups: mothers $\leq$ 28 years old, mothers 29-34 years old and mothers $\geq$ 35 years old. SCT and OC use were categorized into 1 (user) or 0 (non-user). Nondisjunction at meiosis was categorized into 1 (MII NDJ) or 0 (MI NDJ). A new variable of case (1) vs control (0) was created.

2.1.4 Statistical methods

All the analyses were done using the statistical methods implemented in STATA (version 9).

i) The first analysis was to see if the maternal environmental factors are associated with case/control status. All 183 DS cases and 195 controls were included in the analysis. Case vs control was used as the outcome variable for logistic regression modeling. Since this study is an age-matched case-control study, maternal ages were stratified first. That is, in each maternal age category, separate modeling was done to measure correct associations. Independent variables were SCT and OC. Interactions between predictors were included in the models and significant interactions were chosen in the final model.

ii) The second analysis was to see if MI vs MII error in DS cases is associated with environmental factors during the periconceptional period. For these analyses, only 183 DS cases were included in the logistic regression model. The outcome variable was MI vs MII error and predictor variables were SCT, OC and maternal age categories. Various interactions among predictors were also tested and significant interactions were selected for the final model.

iii) The third analysis was focused on recombination events within the MI NDJ group. The number of recombination events was 0, 1 or 2 in MI cases. For this analysis, the events 1 and 2 were aggregated and compared to the cases with no event. A total of 143 DS cases with MI NDJ were included in the logistic regression model. Again, the predictor variables were SCT, OC, and maternal age categories. Various interactions among variables were tested and significant interactions were incorporated in the final model.

iv) The fourth analysis was to find if the position of single chiasma in MI NDJ cases is associated with the environmental factors. Thus, only 32 cases which were caused by MI NDJ and have single chiasma were included in this analysis. The outcome variable was the chiasma position and predictor variables were SCT, OC and maternal age categories. Linear regression modeling methods were used and various interactions among predictors were also tested.

v) The fifth analysis was about the position of single chiasma in MII error cases. A total of 29 DS cases with MII NDJ and with single chiasma were included in this analysis. As in the previous model, single chiasma position was used as the outcome variable and SCT, OC and maternal age categories were used as predictor variables. Linear regression modeling was used. Any significant interactions were also checked.

2.3 RESULTS

2.3.1 Frequencies of Case MI/MII Groups and Control Groups by Age Categories

The study subjects are total 183 DS families and 195 non-DS control families. As shown in Table 2.2, maternal ages in conception are categorized into three groups: young (less than 29 years old), middle (29 to 34 years old) and old (over 34 years old). Overall age distributions in both groups are relatively evenly matched (young/middle/old: 76/59/48 in cases and 59/72/64 in controls: Pearson chi-square=0.069).

However, the proportions of SCT users in the two groups are significantly different (ever/never use: 111/72 in cases and 77/118 in control: Pearson chi-square<0.001). A high proportion of cases have used SCT during the periconceptional period. Proportions of OC users in the two groups are also significantly different (ever/never use: 59/124 in cases and 33/162 in controls: Pearson chi-square=0.001). Again, the case group has a higher number of OC users than the control group.

In MI vs MII NDJ of cases, the proportion of SCT users is not significantly different (ever/never use: 83/60 in MI and 28/12 in MII: Pearson chi-square=0.171). The proportion of OC users is also non-significant (ever/never use: 42/101 in MI and 17/23 in MII: Pearson chi-square=0.116).

Proportions of both SCT and OC users among cases are significantly higher in the old group than in the young or middle age groups (ever/never use of both SCT and OC: 10/66 in young, 12/47 in middle and 18/30 in old: Pearson chi-square=0.006). The disproportion is significant in MI NDJ groups among cases but not in MII NDJ groups. However, the control group has no significant differences in proportions of both SCT and OC users among age groups (ever/never use of both SCT and OC: 5/54 in young, 5/67 in middle and 3/61 in old: Pearson chi-square=0.697).

Table 2.2: Summary of proportion of SCT and OC users in each maternal age category

	Cases				Cases total (n=183)		Controls (n=195)	
	MI (n=143)		MII (n=40)					
Maternal SCT use								
	Never use *(n=60)*	***Ever use*** *(n=83)*	***Never use*** *(n=12)*	***Ever use*** *(n=28)*	***Never use*** *(n=72)*	***Ever use*** *(n=111)*	***Never use*** *(n=118)*	***Ever use*** *(n=77)*
Young (≤28 years)	0.35 (21)	0.49 (41)	0.08 (1)	0.46 (13)	0.31(22)	0.49 (54)	0.31 (37)	0.29 (22)
Middle (29-34 years)	0.43 (26)	0.23 (19)	0.42 (5)	0.32 (9)	0.43 (31)	0.25 (28)	0.36 (43)	0.38 (29)
Old (≥35 years)	0.22 (13)	0.28 (23)	0.5 (6)	0.22 (6)	0.26 (19)	0.26 (29)	0.33 (38)	0.33 (26)
Maternal OC use								
	Never use (*n=101*)	***Ever use*** *(n=42)*	***Never use*** *(n=23)*	***Ever use*** *(n=17)*	***Never use*** *(n=124)*	***Ever use*** *(n=59)*	***Never use*** *(n=162)*	***Ever use*** *(n=33)*
Young (≤28 years)	0.52 (53)	0.21(9)	0.44 (10)	0.24 (4)	0.51 (63)	0.22 (13)	0.3 (49)	0.3 (10)
Middle (29-34 years)	0.3 (30)	0.36 (15)	0.39 (9)	0.29 (5)	0.31 (39)	0.34 (20)	0.37 (60)	0.36 (12)
Old (≥35 years)	0.18 (18)	0.43 (18)	0.17 (4)	0.47 (8)	0.18 (22)	0.44 (26)	0.33 (53)	0.34 (11)
Both SCT and OC use								
	Never use (*n=116*)	***Ever use*** *(n=27)*	***Never use*** *(n=27)*	***Ever use*** *(n=13)*	***Never use*** *(n=143)*	***Ever use*** *(n=40)*	***Never use*** *(n=182)*	***Ever use*** *(n=13)*
Young (≤28 years)	0.47 (55)	0.26(7)	0.41 (11)	0.23 (3)	0.46 (66)	0.25 (10)	0.30 (54)	0.38 (5)
Middle (29-34 years)	0.32 (37)	0.30 (8)	0.37 (10)	0.31 (4)	0.33 (47)	0.30 (12)	0.37 (67)	0.38 (5)
Old (≥35 years)	0.21 (24)	0.44 (12)	0.22 (6)	0.46 (6)	0.21 (30)	0.45 (18)	0.33 (61)	0.24 (3)

2.3.2 Model 1: Case vs Control

Model 1 was to examine whether the maternal environmental factors are associated with DS outcomes. Logistic regression analysis was performed separately in each maternal age category due to the age-matched case-control study design. Table 2.3 shows adjusted odds ratio of DS birth as a function of SCT or OC use. Both SCT use and OC use increase risk of DS birth and interact with maternal age. The effect of SCT use is highest in young mothers (OR=4.17, 95%CI=2.01-8.64), while the middle age group shows minimal effect of SCT use (OR=1.24, 95% CI=0.61-2.52) and the old age group shows moderate effect (OR 2.10, 95% CI=0.92-4.79). However, OC use has the opposite interaction with maternal age groups. In older mothers, OC use has the biggest effect on DS birth (OR 5.53, 95% CI=2.30-13.27), while in the middle age group it has moderate effect (OR 2.50, 95% CI=1.09-5.71) and young mothers were not affected by OC use (OR 0.85, 95% CI=0.33-2.24).

Table 2.3: Association of DS risk with SCT or OC use in each maternal age group, adjusted OR and 95%CI

Characteristic (n=378)		**Young(≤28 years) OR(95%CI)**	**Middle(29-34years) OR(95% CI)**	**Old(>34years) OR(95% CI)**
SCT use	**Never**	**1.0**	**1.0**	**1.0**
	Ever	**4.17 (2.01-8.64)**	**1.24 (0.61-2.52)**	**2.10 (0.92-4.79)**
OC use	**Never**	**1.0**	**1.0**	**1.0**
	Ever	**0.85 (0.33-2.24)**	**2.50 (1.09-5.71)**	**5.53 (2.30-13.27)**

2.3.3 Model 2: MI vs. MII in Cases

Model 2 was a case-only analysis that examined whether MI vs MII non-disjunction in cases is associated with SCT or OC use during periconceptional period (Table 2.4). The risk of MII NDJ relative to MI NDJ is well-known to increase with maternal age, and we did observe that in our dataset (p-value=0.06). SCT is a marginally significant predictor of MII risk (p-value=0.08), increasing risk of MII NDJ compared to non-users and also interacts with maternal age. That is, the effect of SCT use compared to non-users is biggest in young mothers, then in middle age mothers, and smallest in old mothers (p-value=0.045 for interaction). However, OC use does not affect MII risk.

Table 2.4: Association of MII NDJ relative to MI NDJ with SCT or OC use by maternal age, baseline (*) is young mothers (agecat_1) who never use SCT or OC, agecat_2 (middle age group) and agecat_3 (old age group)

Logistic regression output:

Outcome: MII	Odds Ratio	Std. Err.	z	P>|z|	[95% Conf.	Interval]
sct	6.532622	7.010395	1.75	0.080	.7973192	53.5233
agecat_2	3.795402	4.314405	1.17	0.241	.4089389	35.2255
agecat_3	8.447687	9.659997	1.87	0.062	.8982188	79.44993
sctXage_2	.3484773	.4352556	-0.84	0.399	.0301304	4.030358
sctXage_3	.077899	.0990473	-2.01	0.045	.0064453	.941501
oc	1.641257	.6551765	1.24	0.215	.7505576	3.588966

Characteristic (cases: n=183)		Young(≤28 years) OR(95%CI)	Middle(29-34years) OR(95%CI)	Old(>34years) OR(95%CI)
SCT use	Never	1.0*	3.79(0.41-35.2)	8.44(0.89-79.4)
	Ever	6.53(0.79-53.5)	8.64(0.98-75.7)	4.3(0.46-40.1)

2.3.4 Model 3: Recombination Frequency in MI NDJ Group

Model 3 was focused on the recombination frequency in the MI NDJ group among cases. Since the altered recombination pattern is one of the proven risk factors for DS, we wanted to see if the recombination frequency in cases is associated with the maternal environmental factors, especially, if the lack of recombination in MI NDJ is associated with use of SCT or OC. Table 2.5 shows the results of the logistic regression of the binary recombination outcome (zero recombination vs single or more recombination). Both SCT use and OC use are strong predictors for recombination (p-value=0.007, 0.011, respectively). Maternal SCT use interacts with maternal age and is associated with lack of recombination in the young and middle age groups, while older mothers are not affected significantly by SCT use. However, OC use is associated with greater likelihood of recombination regardless of maternal age.

Table 2.5: Association of recombination within MI NDJ with SCT or OC use by maternal age, baseline (*) is young mothers (agecat_1) who never use SCT or OC

```
Logistic regression output:
------------------------------------------------------------------------------
Outcome:     |
recombination| Odds Ratio   Std. Err.      z    P>|z|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
      sct    |    .148857   .1055821    -2.69   0.007     .0370702    .5977425
   agecat_2  |   .9724212   .6013949    -0.05   0.964      .289353    3.267991
   agecat_3  |   .9103459   .6891795    -0.12   0.901     .2064441    4.014306
  sctXage_2  |   1.304147   1.353059     0.26   0.798     .1706871    9.964428
  sctXage_3  |   14.56915   14.92394     2.62   0.009     1.956609    108.4837
      oc     |   3.146814   1.423791     2.53   0.011     1.296405    7.638381
------------------------------------------------------------------------------
```

Characteristic (MI: n=143)		Young(≤28 years)			Middle(29-34years)			Old(>34years)		
		zero recombination	1< recombination	OR (95%CI)	zero recombination	1< recombination	OR (95% CI)	zero recombination	1< recombination	OR (95% CI)
SCT use	Never	13	8	1.0*	15	11	0.97 (0.29-3.27)	7	6	0.91 (0.21-4.0)
	Ever	37	4	0.15 (0.04-0.59)	16	3	0.19 (0.04-0.95)	8	15	1.97 (0.54-7.2)
OC use	Never	45	8	1.0*	23	7	0.97 (0.29-3.27)	8	10	0.91 (0.21-4.0)
	Ever	5	4	3.14 (1.29-7.6)	8	7	3.06 (0.75-12.5)	7	11	2.86 (0.61-13.4)

2.3.5 Model 4: Single Chiasma Position within MI NDJ Group

Model 4 was to investigate if the location of single recombination is associated with SCT or OC use, since the single telomeric recombination is a known risk factor for MI NDJ. As described in Methods, a total of 14 STR markers were used for mapping the position of chiasmata in this study. So the intervals are aligned from centromeric to telomeric, denoted as 1 to 14, and chiasma positions were determined by scoring changes of status of successive markers (N to R or R to N). To examine the association of the single chiasma position with SCT and OC use, only MI NDJ subjects having single chiasma were included in this analysis and all others (no chiasma or more than one chiasma) were excluded. The linear regression analyses of chiasma intervals were run with predictors, SCT, OC and maternal age. Neither SCT nor OC use was significantly associated with the location of recombination in MI NDJ (Table 2.6).

Table 2.6: Association of position of single chiasma within MI NDJ with SCT or OC use by maternal age, agecat_2 (middle age group) and agecat_3 (old age group)

Linear regression output: (n=32)

Outcome: interval	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
sct	.2976961	1.187914	0.25	0.804	-2.139703	2.735095
agecat_2	-1.690914	1.257675	-1.34	0.190	-4.27145	.8896226
agecat_3	-2.21693	1.310208	-1.69	0.102	-4.905255	.471395
oc	-1.486765	1.08804	-1.37	0.183	-3.719239	.7457076
intercept	11.35804	.9466678	12.00	0.000	9.415642	13.30045

2.3.6 Model 5: Single Chiasma Position within MII NDJ Group

Model 5 was to investigate single chiasma position in the MII NDJ group similarly as in Model 4. The linear regression analyses of chiasma intervals shows strong age dependency of the chiasma position (Table 2.7). As mothers get older, the single chiasma position in DS children has tendency towards the pericentromere (p-value<0.01). However, maternal environmental factors, either SCT or OC, are not significantly associated with single chiasma position in MII NDJ.

Table 2.7: Association of position of single chiasma in MII NDJ with SCT or OC use by maternal age

```
Linear regression output:
------------------------------------------------------------------------------
 Outcome:    |
   interval  |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
       sct   |  -1.170564   .8241829    -1.42   0.168    -2.871594    .5304656
    agecat_2 |   -4.81597   .7710536    -6.25   0.000    -6.407346   -3.224594
    agecat_3 |  -7.745754   .9187214    -8.43   0.000    -9.641902   -5.849607
        oc   |   1.042427   .7413865     1.41   0.173    -.4877198    2.572573
   intercept |   11.60558   .8883038    13.06   0.000     9.772212    13.43895
------------------------------------------------------------------------------
```

Characteristic (Single recombinants in MII NDJ: n=29)		Young(≤28 years)		Middle(29-34years)		Old(>34years)	
		Cases	Interval (95% CI)	Cases	Interval (95% CI)	Cases	Interval (95% CI)
SCT use	Never	0	11.61(9.77-13.44)	4	6.79(5.51-8.07)	5	3.86(2.60-5.12)
	Ever	9	10.44(9.36-11.51)	6	5.62(4.22-7.02)	5	2.69(0.84-4.54)
OC use	Never	7	11.61(9.77-13.44)	7	6.79(5.51-8.07)	4	3.86(2.60-5.12)
	Ever	2	12.65(9.87-14.00)	3	7.83(5.73-9.93)	6	4.90(3.28-6.53)

2.4 DISCUSSION

Down syndrome is a well-known genetic birth defect. The major genetic factor is chromosome 21 trisomy due to meiotic non-disjunction. Risk factors for non-disjunction are not much understood yet except for maternal age. However, fewer than 20% of DS children are born to mothers older than 35 years, so it has been suggested that there might be environmental factors affecting chromosome segregation in the ovary. This study focused on maternal behavioral factors in Down syndrome cases. It is a case-control study in an Indian population. Genotypically confirmed DS cases were collected from hospitals in Kolkata, India and the information about maternal periconceptional behaviors was collected retrospectively. Age-matched controls were also collected from same hospitals and so they are matched demographically to the cases. Two maternal behaviors, smokeless chewing tobacco use (SCT) and oral contraceptive use (OC) were studied here. These behaviors in this cohort are quite different than in other studies ((for example, Chen et al., 1999, Kline et al., 1993, Yang et al., 1999) because exposure of SCT in this cohort starts very early in life, usually in teens, the exposure dose of nicotine and other toxic materials in SCT could be higher than in cigarette smoking and oral contraceptive was used irregularly throughout their sexual life, even after conception and generally in high dose. Thus, this cohort could be valuable for studying such environmental factors in DS.

The proportions of SCT users and OC users are significantly higher in cases than in controls. The logistic regression model (model 1) shows that SCT users have a significant high risk for having a DS child. The risk interacts with maternal ages. The young mothers using SCT are likely to have DS child by about four fold compared to the same aged mothers without using SCT. However, the relative risk due to SCT becomes less in middle or old aged mothers. Since absolute risk is much higher in older mothers, this suggests that SCT use might affect adversely the chromosome segregation in a maternal age-independent way. OC users also have significant increase of risk for having a DS child and the effect is also age-related, but in the opposite direction. The old mothers have the highest relative risk of about

five fold due to OC use compared to the same aged non-users, while young or middle aged mothers have little or no OC-related risk. Therefore, the effect of OC use is clearly age dependent, and seems to aggravate the age-effect on the chromosome 21 non-disjunction. This result does not correspond to the other study (Martinez-Frias et al, 2001) where the adverse effect of long-term OC use is high in young and middle aged women. This discrepancy may be due to the specialty of this cohort in the pattern of OC use, again, the irregular use of high dose OC.

The SCT or OC use also affects the pattern of meiotic chromosome non-disjunction, that is, meiosis I vs. meiosis II. Among non-users, older age increases the relative frequency of MII type non-disjunction. However, SCT use increases the relative risk of MII type non-disjunction even in young mothers. As mothers get older, the effect of SCT use is decreased (model 2). Therefore, SCT use seems to work age-independently on the chromosome segregation, which is consistent with the results from model 1. However, OC use has no significant effect on meiosis pattern once maternal ages are controlled in the model.

Lack of recombination has been shown in chromosome non-disjunction in DS (Lamb et al., 1996, 1997, Ghosh et al., 2009). Model 3 shows that the SCT use is strongly associated with lack of recombination in MI type chromosome non-disjunction. The SCT effect is high in young and middle aged mothers, while it is not significant in old mothers. Again, this suggests that SCT use could be an age-independent risk factor for lack of recombination as it was in the previous models. However, the OC use is positively correlated with recombination in the MI NDJ group.

In addition to the absence of recombination, altered recombination patterns have been shown as risk factors in DS. That is, a single telomeric recombination is associated with MI NDJ and a pericentromeric recombination is associated with MII NDJ (Lamb et al., 1996, 1997, Ghosh et al., 2009). Our linear regression model of single chiasma position in MI NDJ group was not predicted by SCT use, OC use or maternal ages. However, the model in MII NDJ shows that the chiasma position has strong age

dependency (model 5). As mothers get older, the single chiasma position in MII NDJ children moves toward the pericentromere. This result corresponds to the previous studies (Lamb et al., 1996, 1997, Ghosh et al., 2009). Nevertheless, SCT or OC use does not affect significantly the position.

Beyond the individual effect of either SCT use or OC use on DS cases, there seems to be a combined effect by co-users of both SCT and OC. As shown in the table 2, there is a significant high proportion of combined users in old mothers of cases compared to same aged mothers in controls. This type of trend has also been shown in another paper (Yang et al., 1999). While the SCT use has age-independent effect on DS, the effect of OC use seems to get exacerbated by the presence of SCT use, especially in old mothers.

In conclusion, our results suggest that chromosome 21 non-disjunction in Down syndrome cases is caused by complex interactions among environmental risk factors and genetic and molecular factors in the ovary. In particular, maternal SCT or OC use has complicated effects that interact with and possibly cause chromosome segregation errors. However, the results should be applied cautiously to other populations because this study cohort is very special as discussed earlier and the sample size is relatively small. Nevertheless, it is a very first study of interactions among all the best-established risk factors for Down syndrome cases of chromosome 21 non-disjunction: maternal age, abnormal recombination, and maternal tobacco use and oral contraceptive use.

3.0 ASSOCIATION OF MATERNAL TELOMERE LENGTH WITH ENVIRONMENTAL RISK FACTORS IN CHROMOSOME 21 NON-DISJUNCTION

3.1 INTRODUCTION

As an initial step for investigating molecular mechanisms of maternal aging effect on Down syndrome, maternal telomere length has been studied previously (Ghosh et al., 2010). The study found that the length of telomere shortens by function of ages and the loss is higher in cases than in control. In this thesis, association of maternal environmental factors, periconceptional smokeless chewing tobacco use and oral contraceptive use, with telomere length was studied in Down syndrome cases.

3.2 METHODS

3.2.1 Subjects and genotyping

All procedures for selecting subjects and genotyping are same as in previous chapter. Total 206 cases were collected for this study. 17 STR markers were used for genotyping.

3.2.2 Determination of telomere length

All blood samples were taken within a week of birth of the child in cases. DNA was prepared from the blood samples and restriction-digested and separated on an agarose gel. The length of the telomere was analyzed by Southern blot techniques using the kit, TeloTAGGG Telomere Length Assay from Roche chemicals. The calculation of the length took into account the higher signal intensity from larger telomere repeats because of multiple hybridization of the telomere-specific probe. The procedures were performed blinded to MI or MII origins.

3.2.3 Data management

As in previous chapter, data from Dr. Ghosh were stored on a Microsoft Excel file. Recorded variables for 206 DS cases are telomere length, meiosis I/II error, maternal age at conception, SCT chewer, oral contraceptive (OC) use, mapping of 17 STR markers. Using the STR map, recombination events and chiasma positions were scored in the Excel file. Then the data was transferred into STATA software. Fifty eight subjects had no telomere length measurement, so only 148 subjects were included for the analyses. As in previous chapter, maternal age was categorized into three groups: mothers $\leq$ 28 years old, mothers 29-34 years old and mothers $\geq$ 35 years old. SCT and OC use were categorized into 1 (user) or 0 (non-user). Nondisjunction at meiosis was categorized into 1 (MII error) or 0 (MI error).

3.2.4 Statistical methods

Since the goals of the study is to look whether the telomere length of the mothers of Down syndrome cases is associated with the maternal periconceptional behaviors, linear regression methods were used with the telomere length estimation as outcome variable. The behavior factors of SCT use and OC use, and maternal age and MI or MII NDJ were incorporated as covariates.

3.3 RESULTS

3.3.1 Frequencies of SCT or OC Users in Age Categories and in MI and MII NDJ Groups

The study subjects are total 148 DS families which were confirmed cases as maternal NDJ by genotyping, have complete information of maternal periconceptional behaviors and have measured for telomere length. As shown in Table 3.1, maternal ages in conception are categorized into three groups; young (less than 29 years old), middle (29 to 34 years old) and old (over 34 years old). Age distributions are: 56/43/49 (young/middle/old).

Proportions of SCT users among age groups are ever/never use: 38/18 in young and 22/21 in middle and 26/23 in old (Pearson chi-square=0.169). However, proportions of OC users among age groups are

significantly different (ever/never use: 9/47 in young and 15/28 in middle and 28/21 in old: Pearson chi-square=0.000). That is, the old group has significantly high proportion of OC users.

In MI vs MII NDJ groups, proportion of SCT users is not significantly different (ever/never use: 60/51 in MI and 26/11 in MII: Pearson chi-square=0.083). Proportion of OC users is also non- significant (ever/never use: 37/74 in MI and 15/22 in MII: Pearson chi-square=0.426).

Proportions of both SCT and OC users are significantly higher in old group than in young or middle age groups (ever/never use of both SCT and OC: 7/49 in young, 11/32 in middle and 18/31 in old: Pearson chi-square=0.015).

Table 3.1: Summary of proportion of SCT and OC users in each maternal age category

	MI (n=111)		**MII (n=37)**		**All cases (n=148)**	
Maternal SCT use						
	Never use *(n=51)*	***Ever use*** *(n=60)*	***Never use*** *(n=11)*	***Ever use*** *(n=26)*	***Never use*** *(n=62)*	***Ever use*** *(n=86)*
Young (≤28 years)	0.33 (17)	0.42 (25)	0.09 (1)	0.5 (13)	0.29 (18)	0.44 (38)
Middle (29-34 years)	0.35 (18)	0.23 (14)	0.27 (3)	0.31 (8)	0.34 (21)	0.26 (22)
Old (≥35 years)	0.32 (16)	0.35 (21)	0.64 (7)	0.19 (5)	0.37 (23)	0.3 (26)
Maternal OC use						
	Never use (*n=74*)	***Ever use*** *(n=37)*	***Never use*** *(n=22)*	***Ever use*** *(n=15)*	***Never use*** *(n=96)*	***Ever use*** *(n=52)*
Young (≤28 years)	0.47 (35)	0.19 (7)	0.55 (12)	0.13 (2)	0.49 (47)	0.17 (9)
Middle (29-34 years)	0.29 (21)	0.30 (11)	0.32 (7)	0.27 (4)	0.29 (28)	0.29 (15)
Old (≥35 years)	0.24 (18)	0.51 (19)	0.13 (3)	0.6 (9)	0.22 (21)	0.54 (28)
Both SCT and OC use						
	Never use (*n=85*)	***Ever use*** *(n=26)*	***Never use*** *(n=27)*	***Ever use*** *(n=10)*	***Never use*** *(n=112)*	***Ever use*** *(n=36)*
Young (≤28 years)	0.42 (36)	0.23 (6)	0.48 (13)	0.10 (1)	0.44 (49)	0.19 (7)
Middle (29-34 years)	0.3 (25)	0.27 (7)	0.26 (7)	0.4 (4)	0.28 (32)	0.31(11)
Old (≥35 years)	0.28 (24)	0.5 (13)	0.26 (7)	0.5 (5)	0.28 (31)	0.5 (18)

3.3.2 Linear Regression Models of Telomere Length

Figure 3.1 shows the relationship of telomere length with maternal age in the regression model. As shown in previous studies (Ghosh et al., 2010), the telomere length is linearly decreasing as function of age and the p-value is highly significant (0.0001).

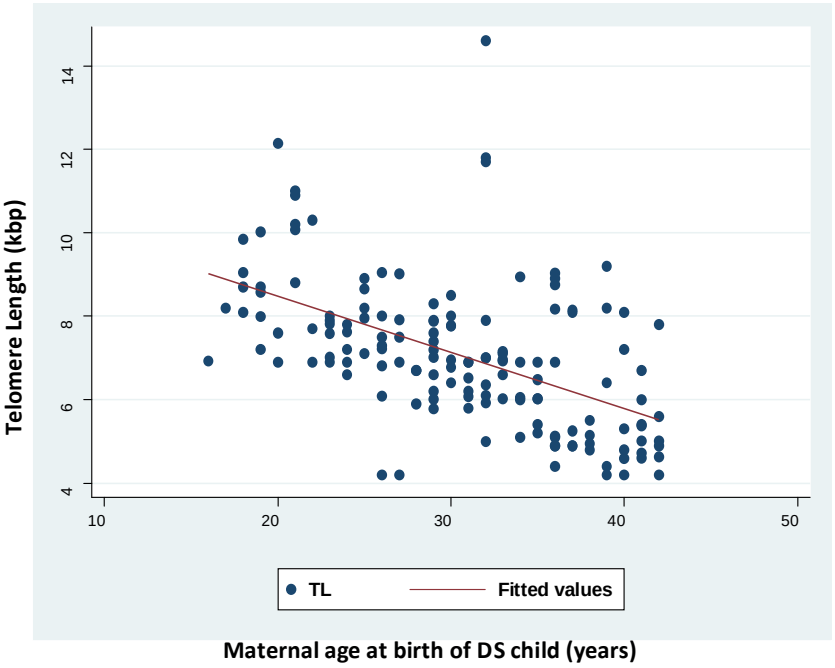

Figure 3.1: Relationship of telomere length with maternal age

The previous study by Ghosh et al. (2010) has also shown the association of telomere length with types of MI or MII NDJ. In this dataset, telomere length is also shorter in MII NDJ group than in MI NDJ group (mean of MI=7.25, 95%CI (6.95, 7.55): mean of MII=6.5, 95%CI (5.78, 7.21): p-value of t-test=0.0257).

To investigate association of maternal behavioral risk factors with telomere length, the covariates of SCT and OC as well as maternal age and MI/MII were included in the model. SCT use is a significant predictor for the model, while OC use is not (regression p-values=0.001, 0.175, respectively). There is a significant interaction between MI/MII NDJ and SCT use (regression p-value=0.037). The final model for association is shown in Table 3.2. As shown in the table, the model predicts that the overall length of

telomere is shorter in older age than in younger age. MII NDJ group has shorter telomere length than MI NDJ group. SCT use decreases the telomere length in MI NDJ group, regardless of age groups but MII NDJ group is not affected by SCT use.

Table 3.2: Association of telomere length with SCT use, OC use and MI/MII by maternal age

```
Linear regression output:
------------------------------------------------------------------------------
Telomere_
      length |      Coef.   Std. Err.      t    P>|t|     [95% Conf. Interval]
-------------+----------------------------------------------------------------
    agecat_2 |  -.7101924   .2998666    -2.37   0.019    -1.302972   -.1174128
    agecat_3 |  -1.966582   .2944688    -6.68   0.000    -2.548691   -1.384473
     MII     |  -1.467714   .4942638    -2.97   0.004     -2.44478   -.4906475
     sct     |  -1.229833   .2806554    -4.38   0.000    -1.784636   -.6750305
    MIIxsct  |   1.282267   .6091442     2.11   0.037     .0781039     2.48643
   Intercept |   8.774682    .265843    33.01   0.000      8.24916    9.300203
------------------------------------------------------------------------------
```

Characteristic			**Young(≤28 years)**	**Middle(29-34years)**	**Old(>34years)**
			Telomere-length (95% CI)	**Telomere-length (95% CI)**	**Telomere-length (95% CI)**
MI	**SCT use**	**Never**	**8.77(8.25-9.30)**	**8.06(7.53-8.59)**	**6.81(6.27-7.35)**
		Ever	**7.55(7.07-8.02)**	**6.83(6.28-7.39)**	**5.58(5.07-6.08)**
MII	**SCT use**	**Never**	**7.31(6.32-8.30)**	**6.59(5.63-7.57)**	**5.34(4.45-6.24)**
		Ever	**7.36(6.74-7.98)**	**6.65(5.97-7.33)**	**5.39(4.69-6.10)**

3.4 DISCUSSION

Since the maternal age is the most evident risk factor of DS, molecular mechanisms causing chromosome non-disjunction in an aged ovary has been speculated for a long time. So, the theory of "biological aging" has been proposed (Warbuton, 2005). That is, the biological ages of the women bearing DS children could be different to those of control women of the same chronological age. One of the supporting studies is that women with trisomic pregnancies have earlier menopause than control women (Kline et al. 2000). Recently, the telomere length has been shown to be shortened replicatively

in aging cells and was considered as a molecular marker for estimating cellular aging (Benetos et al. 2001; Cawthon et al. 2003; Aviv 2008). Nevertheless, studies have not found any evidence of premature telomere shortening in women bearing a DS child. For example, a study by Dorland et al. (1998) did not find any significant difference of telomere length between young mothers bearing a live born DS child and age-matched control mothers.

The previous study by Ghosh et al. (2010) was designed as a cross-sectional study for measuring telomere length of women at the time of birth of either DS child or euploid child in Indian population. Ages of the subjects were from 18 years to 42 years and the groups were MI NDJ, MII NDJ and control. The study showed that the telomere length decreases with ages in all groups and the loss of the length is biggest in MII NDJ, then in MI NDJ and least in control. When the telomere length is stratified by age, all young mothers have similar telomere length regardless of groups. However, the old mothers (35-42 years old) among groups have significantly different telomere length (control/MI/MII groups: $7.9\pm0.9/6.1\pm1.3/4.7\pm0.3$ kbp). The p-values of the pair-wise comparisons between control and MI NDJ, between control and MII NDJ, and between MI NDJ and MII NDJ are <0.001, <0.001 and 0.004, respectively. Thus, the study clearly shows that the older mothers bearing DS child have genetically older than the age-matched control mothers. It suggests that the factors accelerating telomere loss in advanced reproductive age might also affect the chromosome segregation in oocyte. Those factors could be age-related environmental changes in ovary. In addition, the fact that the MII NDJ has biggest loss of the telomere length is intriguing because the old mothers have higher risk of MII NDJ.

This chapter is an extension of the previous study by Ghosh et al. (2010). Here we explore the association of the maternal environmental factors with telomere lengths of women bearing DS child. As in the chapter 1, the study cohort is special in terms of exposure of the environmental factors which are smokeless chewing tobacco use (SCT) and oral contraceptive use (OC). They have been exposed very

early age in life and the exposure dose has been high and irregular compared to other Western population.

This analysis does not include a control group and focuses on MI NDJ vs. MII NDJ. The linear regression model shows that the telomere length is decreased by ages similar to the result in the previous study. MII NDJ has a shorter mean telomere length than MI NDJ, which, again, is similar to the result as the previous study. When SCT use and OC use were incorporated in the model, SCT use affects the telomere length, while OC use does not. The effect of SCT use interacts significantly with MI NDJ, so SCT users have shorter telomere length than SCT non-users. The effect size is similar throughout the age groups, indicating that the SCT use works age-independently on telomere length shortening. However, the telomere length of MII NDJ group is not affected by SCT use. Regardless of SCT use, the telomere length in MII NDJ is even shorter than in SCT users of MI NDJ, indicating that in MII NDJ, especially in older mothers the age-related factors seem to be dominant causes in shortening telomere length.

In summary, this study shows that the telomere length decreases with aging in women bearing DS child, MII type NDJ has shorter telomere length than MI type NDJ, and the SCT use accelerates the shortening of telomere length in MI type NDJ. Thus, this study provides for the first time an evidence of that maternal behavioral factors affect molecular aging. However, it has to keep in mind that this study cohort is extraordinary in terms of exposure of such environmental factors.

4.0 CONCLUSION

This thesis focused on the statistical analyses of two genetics datasets from Indian Down syndrome case-control study. Both studies were designed for studying association of maternal environmental factors, smokeless chewing tobacco use and oral contraceptive use, in Down syndrome. By using logistic and linear regression analysis methods as well as basic statistical tools, I found that both behaviors during periconceptional period are significant risk factors for DS. The effects appear on the pattern of the chromosome 21 non-disjunction as well as on the chromosomal recombination frequency and position. And there are interactions of these factors with maternal ages. Thus, the risk of Down syndrome due to maternal smoking appears higher on younger mothers than on older mothers. These factors also associate with telomere length of mothers bearing DS child. Smokers have shorter telomere than non-smokers. From this thesis research, I have had good experiences for managing, processing and analyzing population genetics datasets. Most of all, I have learned about how to choose analysis variables and how to interpret the analysis results. And I also learned that the study design as well as study population also have to be considered in the analysis procedure and interpretation.

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