PRELIMINARY COMMUNICATION

Chromosomal Instability in Amniocytes From Fetuses of Mothers Who Smoke

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Context Tobacco increases the risk of systemic diseases, and it has adverse effects on pregnancy. However, only indirect data have been published on a possible genotoxic effect on pregnancy in humans.

Objectives To determine whether maternal smoking has a genotoxic effect on amniotic cells, expressed as an increased chromosomal instability, and to analyze whether any chromosomal regions are especially affected by exposure to tobacco.

Design, Setting, and Patients In this prospective study, amniocytes were obtained by routine amniocentesis for prenatal diagnosis from 25 controls and 25 women who smoke (≥10 cigarettes/d for ≥10 years), who were asked to fill out a smoking questionnaire concerning their smoking habits. Chromosomal instability was analyzed in blinded fashion by 2 independent observers in routine chromosome spreads. Breakpoints implicated in chromosomal abnormalities were identified by G-banding.

Main Outcome Measures Association between maternal smoking and increased chromosomal instability in amniotic fluid cells, expressed as chromosomal lesions (gaps and breaks) and structural chromosomal abnormalities.

Results Comparison of cytogenetic data between smokers and nonsmokers (controls) showed important differences for the proportion of structural chromosomal abnormalities (smokers: 12.1% [96/793]; controls: 3.5% [26/752]; $P=.002$) and to a lesser degree for the proportion of metaphases with chromosomal instability (smokers: 10.5% [262/2492]; controls: 8.0% [210/2637]; $P=.04$), and for the proportion of chromosomal lesions (smokers: 15.7% [391/2492]; controls: 10.1% [267/2637]; $P=.045$). Statistical analysis of the 689 breakpoints detected showed that band 11q23, which is a band commonly implicated in hematopoietic malignancies, was the chromosomal region most affected by tobacco.

Conclusions Our findings show that smoking 10 or more cigarettes per day for at least 10 years and during pregnancy is associated with increased chromosomal instability in amniocytes. Band 11q23, known to be involved in leukemogenesis, seems especially sensitive to genotoxic compounds contained in tobacco.

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THE LONG-TERM PUBLIC HEALTH consequences of regular tobacco consumption include an increased risk of coagulation problems, cancer, cardiovascular disease, chronic obstructive pulmonary disease, and adverse effects on pregnancy. Maternal smoking during pregnancy has many consequences both during and after pregnancy, such as infertility, coagulation problems, obstetric accidents such as extrauterine pregnancy or placenta previa, and intrauterine growth retardation.[1] A relationship between postnatal exposure to tobacco and childhood cancer, especially leukemia and lymphomas, has also been suggested.[2]

Tobacco contains a high number of mutagenic compounds.[3] Recently, the presence of tobacco-specific metabolites has been described in fetal blood and cell-free amniotic fluid (transferred from the mother via placenta) and in newborns from women who smoke,[4-6] suggesting a possible genotoxic effect of smoking during pregnancy. However, although many cytogenetic studies have demonstrated the existence of an increased incidence of chromosomal aberrations, sister chromatid exchanges (SCEs), micronuclei, and fragile-site expression in peripheral blood lymphocytes of adult smokers,[7-10] no data regarding a possible genotoxic effect of tobacco on the embryo and fetus are available. Only indirect data using chorionic villi have been published[11,12]; in one case, an increase in SCEs was found in direct preparations,[11] while in the other, chromosomal lesions were not increased.[12]

In this study we assess the possible genotoxic effect of maternal smoking on amniotic fluid cells, based on the presence of an increased chromosomal instability expressed as chromosomal lesions (gaps and breaks) and structural chromosomal abnormalities. We also analyze whether any chromosomal regions are especially affected by exposure to tobacco in the fetus.

See also p 1264 and Patient Page.

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METHODS

Patients

In this prospective study, amniocytes were obtained by amniocentesis for prenatal diagnosis. The study group consisted of 25 women smokers and 25 nonsmoking women between the 13th and 26th postmenstrual week. Women were first personally interviewed at length by one author (I.R.) regarding their consumption of alcohol, coffee, and tea. Only if the answers were negative were women asked to fill out the smoking questionnaire concerning their current and previous smoking habits, those of their husbands, and smoking in their occupational setting. Smokers had smoked 10 or more cigarettes per day for at least 10 years. Nonsmokers (controls) were not exposed to tobacco at home or at work (ie, no passive smoking). In the smokers group, 5 fathers smoked 5 to 20 cigarettes per day (S2, S5, S7, S8, and S17), 10 fathers were nonsmokers (S1, S3, S12, S13, S15, S16, S18, S20, S23, and S25), and the smoking habits of the rest of the fathers was unknown. The first 25 women who fulfilled all of these conditions and were in good health were included in each group. In total, 800 interviews were carried out. Four hundred ninety-six interviews were required to find the 25 nonsmokers who fulfilled the strict criteria set up in our protocol; 175 interviews were required to find the 25 mothers who had smoked 10 or more cigarettes daily for at least 10 years and who continued smoking during pregnancy. The 129 remaining interviews correspond either to women who smoked fewer than 10 cigarettes per day, those who had smoked for less than 10 years, or those who had quit smoking when they knew they were pregnant.

TABLE 1 and TABLE 2 present data for maternal age, paternal age, number of previous pregnancies, years of maternal smoking before present pregnancy, number of cigarettes smoked per day, weeks of gestation, and the indications for prenatal diagnosis for smokers and controls, respectively. The study was approved by the Universitat Autònoma de Barcelona institutional ethics committee. Informed consent was given in writing by all participants.

Cytogenetic Analysis

The amniotic fluid was centrifuged in 2 different tubes at 800 rpm for 5 minutes at room temperature. The super-

Table 1. General Characteristics of the Mothers Who Smoke

Mother	Maternal Age, y	Paternal Age, y	No. of Previous Pregnancies	Years Maternal Smoking Before Present Pregnancy	Cigarettes/d	Weeks of Gestation	Indications for Prenatal Diagnosis*
S1	35	36	1	16	>20	16	Spontaneous abortion/IVF
S2	33	33	2	13	20	19	Spontaneous abortion
S3	28	38	1	10	20	18	Toxoplasmosis
S4	35	35	4	19	>20	16	Spontaneous abortion
S5	40	49	0	24	20	14	Age
S6	33	39	1	18	>20	15	Anxiety
S7	37	35	1	22	20	14	Age
S8	37	41	3	21	20	15	Age
S9	37	41	2	22	>20	14	Age
S10	37	39	3	24	>20	15	Spontaneous abortion
S11	37	39	0	11	10-15	15	Age
S12	41	40	2	20	20	15	Age
S13	41	40	2	20	>20	15	Age
S14	36	?	1	12	15-20	16	Age
S15	39	40	1	21	15-20	16	Previous fetus with de novo chromosomal alteration/IVF
S16	39	40	1	21	15-20	16	Previous fetus with de novo chromosomal alteration/IVF
S17	38	38	2	23	15-20	15	Age
S18	34	44	1	16	>20	17	Anxiety
S19	37	39	1	22	15-20	16	Age/nephew with Klinefelter syndrome
S20	37	37	1	19	>20	15	Age
S21	32	31	0	18	15-20	16	Age/anxiety
S22	41	32	0	21	10-15	14	Age/IVF
S23	37	38	0	22	15-20	15	Age/triple screening (1/40)/IVF
S24	35	38	3	21	10-15	15	Antecedent of Potter syndrome
S25	43	49	0	25	15-20	16	Age

Abbreviation: IVF, in vitro fertilization.
*Numbers in parentheses indicate triple screening risk scores.

Table 2. General Characteristics of Nonsmoking Controls

Control	Maternal Age, y	Paternal Age, y	No. of Previous Pregnancies	Weeks of Gestation	Indications for Prenatal Diagnosis*
C1	34	36	0	17	IVF (ICSI)
C2	36	36	2	16	Age/spontaneous abortion
C3	34	37	0	15	Anxiety
C4	34	35	3	16	Spontaneous abortion
C5	37	34	1	15	Age/spontaneous abortion
C6	34	32	1	16	Echographic fetal anomalies
C7	34	42	0	16	Triple screening (1/77) + IVF (ICSI)
C8	37	37	4	14	Age
C9	33	36	2	13	Triple screening (1/151)
C10	29	29	0	16	IVF (ICSI)
C11	37	36	1	16	Age
C12	35	44	2	17	Age
C13	28	30	0	16	Triple screening (1/250)
C14	34	30	1	15	Triple screening (1/64)
C15	33	35	1	14	Anxiety
C16	35	?	0	15	IVF (ICSI)
C17	35	35	1	14	Triple screening (1/60)/spontaneous abortion
C18	26	28	0	26	Infection (cytomegalovirus)
C19	30	33	2	16	Spontaneous abortion/IVF
C20	39	41	3	17	Age/spontaneous abortion
C21	37	38	2	16	Age/spontaneous abortion
C22	31	31	2	16	Triple screening (1/188)/spontaneous abortion
C23	36	38	1	15	Age/triple screening (1/85)
C24	31	31	0	23	Echographic signs/IVF
C25	36	36	0	?	Age/triple screening (1/41)

Abbreviations: ICSI, intracytoplasmic sperm injection; IVF, in vitro fertilization.
*Numbers in parentheses indicate triple screening risk scores.

natant was removed under sterile conditions, leaving a pellet in 0.5 mL of amniotic fluid. Cells were resuspended with fresh culture medium. Four cultures were set up: two 35-mm plastic petri dishes containing a 22-mm–square coverslip and 2 flat plastic tubes. The culture medium used for petri dishes was Chang (Irvine Scientific, Santa Ana, Calif) with 1% penicillin-streptomycin (Invitrogen Corp, Carlsbad, Calif). The media used for tubes were RPMI:HAM-F10 (1:1) (Invitrogen) with 5.5% fetal calf serum (Invitrogen); 2.5% ultroser G, which is a substitute for calf bovine serum (Ciphergen Biosystems Inc, Fremont, Calif); 2% L-glutamine (Invitrogen); and 1% penicillin-streptomycin (Invitrogen). Cultures were placed in an incubator with 5% carbon dioxide in ambient air at 37°C, monitored visually, and the medium changed every 2 to 3 days. Petri dishes were used only for prenatal diagnosis. For the present study, cultures from smokers and controls were both first grown in an RPMI: HAM-F10 medium. When cultures in a flat tube showed sufficient growth (≥5 colonies), the cells were distributed into 2 plastic petri dishes containing Chang medium and harvested 24 hours later using an in situ fixation technique; colcemid was added for the last 45 minutes. The medium containing colcemid was replaced by 0.8% sodium citrate at room temperature for 12 to 15 minutes. A few drops of 3:1 methanol/acetic acid fixative were added to the hypotonic solution for 5 minutes. The fixative was replaced with fresh fixative for 20 minutes. One additional fixative change was made. Following removal of the final fixative, the coverslips were allowed to dry under specific humidity conditions (48%-52%).

Preparations were stained with Leishman stain (1:4 in Leishman buffer), coded, and evaluated for the presence of gaps and breaks by 2 authors (R.A.C., C.F.) blinded to participant smoking status. Differences were resolved by discussion and consensus. Location and types of anomaly were recorded by each evaluator and compared at the end of the study. Cytogenetic evaluation was performed according to standard procedures. Only high-quality metaphases were analyzed. About 100 randomly selected metaphases uniformly stained were analyzed in each case. Later, preparations were destained for 1 minute in 3:1 methanol/acetic acid and immediately incubated for 10 to 30 minutes in 2xSSC at 65°C, washed with distilled water, air dried, and stained for 3 minutes with Wright Giemsa stain to identify the bands where the lesions were located. To characterize structural chromosomal abnormalities (deletions, acentric fragments, duplications, translocations, inversions, and marker chromosomes), only high-quality banded metaphases were used; at least 25 banded metaphases per patient were karyotyped.

Statistical Analysis

A generalized estimating equation (GEE)[13] was used for assessing the differences between the smoker and control groups for the different types of chromosomal instability. The GEE approach is an extension of generalized linear models designed to account for repeated within-individual measurements. This technique is particularly indicated when the normality assumption is not reasonable as, for instance, for discrete data. The GEE model was used instead of the classic Fisher exact test because the former takes into account the possible within-fetus correlation, whereas the latter assumes that all observations are independent. Since several metaphases were analyzed per fetus, the GEE model is more appropriate. In addition, this method allows

for the inclusion in the model of additional explanatory variables as covariates. In our analyses, the variance function for the binomial distribution and the logit link function were specified for the model. The response variable was defined as the number of chromosomal anomalies/number of metaphases tested for each fetus.

To identify which chromosome bands could be considered especially affected by the genotoxic effect of tobacco, the fragile site multinomial method (version 995) was used.[14,15] This multinomial statistical method is specifically designed to identify chromosomal fragile sites at loci where chromosome breaks are found. The fragile site multinomial method can be used for a maximum of 30 individuals, and the program performs the analyses for each individual separately and for the data pooled over all individuals. Because the number of chromosomal abnormalities per individual was much lower than the minimum (200 at the 400-band resolution level) required by the program to perform reliable estimates, only results from data pooled over the smoker and control groups were considered. The standardized χ^2 and G^2 tests were used for assessing the statistical significance of the chromosome bands with breaks, gaps, or rearrangements in each group.

To identify the bands with a greater sensitivity (implicated in structural chromosomal abnormalities or in chromosomal lesions) in smokers relative to controls, a variable was computed, defined for each band as the number of gaps and breaks (including those involved in structural abnormalities) in smokers minus their number in controls (difference). Bands with positive values in the computed variable indicated a greater tendency to break in smokers, while bands with negative values suggested the opposite. In addition, those bands with a computed difference value more than 3 SDs from the mean difference were considered extreme values and selected for further analysis with the GEE model. In the particular case of a band presenting a zero value for each of the individuals belonging to one group, alternative analyses such as the Fisher exact test and the nonparametric Wilcoxon rank-sum test (for which exact *P* value computation was requested) were applied.

Statistical significance was set at $P<.05$. Statistical analyses were carried out with SAS/STAT release 8.01 (SAS Institute Inc, Cary, NC). The GEE model was fitted using the REPEATED statement in the GENMOD procedure. The conservative type 3 score statistics were used for the analysis of the model effects.[16]

Table 3. Frequency and Types of Chromosomal Instability in Amniocytes From Fetuses Carried by Smokers and Controls

Variable	Smokers	Controls
Total metaphases analyzed (uniform stain), No.	2492	2637
Total metaphases karyotyped (G-banded), No.	793	752
Chromosomal instability, No./total (%)	262/2492 (10.5)	210/2637 (8.0)
Gaps and breaks, No. (%)	(n = 2492)	(n = 2637)
Total	391 (15.7)	267 (10.1)
Gaps	183 (7.3)	144 (5.5)
Breaks	208 (8.3)	123 (4.7)
Structural chromosomal abnormalities, No./total (%)*	96/793 (12.1)	26/752 (3.5)
Deletions	28	6
Deletions + acentric fragments	29	13
Acentric fragments	7	1
Translocations (+2der)	12	2
Dicentric translocations	5	2
Inversions	2	0
Duplications	1	0
Markers	11	2
Intrachromosomal reorganizations	1	0

*Similar values can be found in Price,[17] with 8% to 16% structural chromosomal abnormalities (total) and in Kerber and Held,[18] with 12.4% to 20.9% structural abnormalities per case.

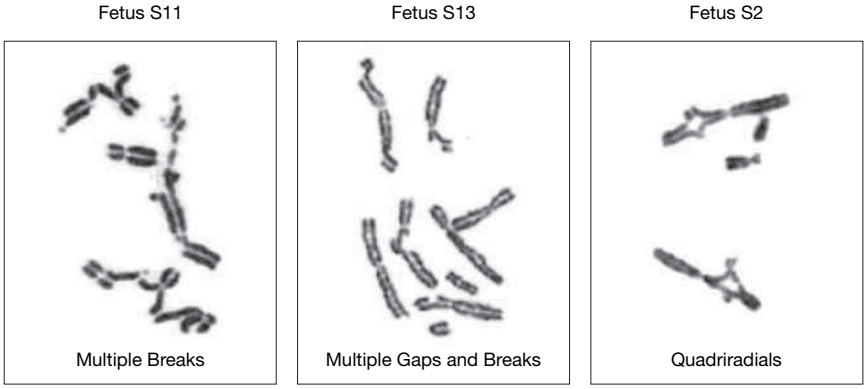
RESULTS

Chromosomal Instability in Amniocytes From Fetuses of Mothers Who Smoke

The number of metaphases with chromosomal instability, the frequency and type of chromosomal lesions, and the frequency of structural abnormalities in amniocytes from fetuses of the smoker and control groups are shown in TABLE 3. The clinical data of the patients (Tables 1 and 2) revealed that the mean maternal age in the smoker group was significantly higher than in the control group. However, the difference (3 years) found in the mean values should not influence a study based on the analysis of lesions and structural abnormalities, because maternal age influences numerical but not structural abnormalities. In this regard, no significant correlation was obtained in our data between any of the above cytogenetic variables and maternal age, within either the smoker or control groups. Nevertheless, because the GEE method used for the analysis allows for the inclusion of continuous explanatory variables as covariates, the contribution of age was considered. Moreover, the results obtained for the whole sample were consistent with those from a particular subset (all women except those who underwent in vitro fertilization or intracytoplasmic sperm injection) in which no significant difference in maternal age between smokers and controls was present. Finally, no differences were found between smokers and controls for the number of weeks of gestation.

First, we used a reduced model in which age was not considered. In all analyses, the smoking effect was significant for chromosomal instability (smokers: 10.5% [262/2492]; controls: 8.0% [210/2637]; $P=.04$), chromosomal lesions (smokers: 15.7% [391/

Figure 1. Partial Metaphases of Amniocytes From Fetuses of Mothers Who Smoke, Showing Spontaneous Chromosomal Instability



Preparation stained with Leishman stain.

2492]; controls: 10.1% [267/2637]; $P=.045$), and to a higher degree for structural chromosomal abnormalities (smokers: 12.1% [96/793]; controls: 3.5% [26/752]; $P=.002$). In both groups, the most frequent structural chromosomal abnormalities were deletions and translocations (Table 3). Deletions (smokers: 7.2% [57/793]; controls: 2.5% [19/752]) and translocations (smokers: 2.1% [17/793]; controls: 0.5% [4/752]) were both also significant ($P=.006$ and $P=.01$, respectively).

Next, a model in which age was included as a covariate was considered. The age effect was not significant for any of the analyses performed (for chromosomal instability, $P=.40$; chromosomal lesions, $P=.16$; structural chromosomal abnormalities, $P=.64$; deletions, $P=.40$; and translocations, $P=.10$). The high P values obtained for maternal age indicate that this factor does not influence the chromosomal anomalies observed and suggest that it could be removed from the model. Nevertheless, the model incorporating maternal age was evaluated. The inclusion of this covariate increased the P values of the smoking factor for all chromosomal anomalies analyzed. A nearly significant increase was observed in the percentage of metaphases with chromosomal instability in amniocytes from smokers compared with those from controls ($P=.05$). The proportion of chromosomal lesions was marginally influential in amniocytes from smokers compared with those from controls ($P=.10$). In the smoker group, 2 cases (S9 and S11) had metaphases with multiple chromosomal lesions or pulverized cells; these metaphases were not included in the estimation of the number of lesions. The much higher incidence of structural chromosomal abnormalities in karyotyped metaphases in the smoker group than in the control group remained significant ($P=.01$). The incidence of deletions was higher in the smoker group than in the control group ($P=.01$), while the incidence of translocations became nonsignificant ($P=.12$). More than one third of the fetuses from mothers who smoke (36% [9/25]) had triradial or quadriradial figures in their metaphases (S2, S5, S9, S10, S11, S13, S15, S19, and S20) (FIGURE 1); in controls, only 1 quadriradial was found (C24).

Finally, 5 smokers and 6 controls had become pregnant by in vitro fertilization or intracytoplasmic sperm injection. To discard a possible effect of the hormonal treatment on the evaluation of the genotoxic effects of tobacco, the statistical analyses were repeated excluding these individuals. It is worth noting that in this subset of women excluding those who had undergone in vitro fertilization or intracytoplasmic sperm injection, the maternal ages of smokers and controls were not statistically different. However, for consistency with the previous analyses, an extended model including maternal age as a covariate and a reduced model not including this factor were considered. Similar results were obtained for both models. In the extended model and as in the results obtained for the whole sample, maternal age showed no significant association with observed chromosomal anomalies. In this extended model, the results for the smoking factor reached statistical significance for both the proportion of metaphases with chromosomal instability (smokers: 10.3% [200/1951]; controls: 7.2% [145/2023]; $P=.03$) and the proportion of structural chromosomal abnormalities (smokers: 13.3% [83/624]; controls: 3.0% [17/570]; $P=.01$) and showed a marginal influence for the proportion of chromosomal lesions (smokers: 15.3% [298/1951]; controls: 9.0% [182/2023]; $P=.08$). The results obtained for the reduced model reached statistical significance for both the proportion of metaphases with chromosomal instability ($P=.02$) and the proportion of structural chromosomal abnormalities ($P=.002$) and showed a nearly significant association for the proportion of chromosomal lesions (smokers: 15.3% [298/1951]; controls: 9.0% [182/2023]; $P=.05$).

Aneuploid metaphases were found in smokers and controls (smokers: 12.5% [99/793]; controls: 10.8% [81/752]) without showing statistical significance between them ($P=.52$ for the reduced model; $P=.36$ for the extended model).

In sum, our results suggest that smoking during pregnancy has a genotoxic effect that is not influenced by maternal age.

Cytogenetic results for each individual are shown in TABLE 4 and TABLE 5. All fetuses had normal constitutional karyotypes (46,XX or 46,XY). A pseudomosaicism (46,XY,83%/46,XY,t[X;1][p22.2;q25]17%) was detected in S16 but not confirmed after birth.

Specific Chromosome Bands Affected by Exposure to Tobacco

The breakpoint distribution of the 430 breakpoints clearly identified by G-

Table 4. Cytogenetic Results in Amniocytes From Fetuses Carried by Mothers Who Smoke

Mother	Karyotype of Fetus	No./Total (%)		Triradials and Quadriradials	Structural Abnormalities, No./Total (%)	Types of Structural Abnormalities*
		Aberrant Metaphases	Cells With Gaps and Breaks			
S1	46,XY	9/103 (8.7)	8/103 (7.8)	. . .	3/43 (7.0)	del(2)(q24),del(2)(q24.3) + ace,del(11)(q13) + ace
S2	46,XY	8/108 (7.4)	12/108 (11.1)	tr(1;2)(q12;p16) qr(2;13)(p23;q13) qr(1;19)(q24;q13.1) tr(11;18)(q23;q11.2)	2/48 (4.2)	del(5)(q31) + ace,der(1)(p32;q22)
S3	46,XX	8/100 (8.0)	7/100 (7.0)	. . .	9/43 (20.9)	t(X;6)(p11.3;q16),del(5)(q15) + ace, del(6)(q13) + ace,del(1)(q12) + ace, del(2)(p13) + ace,del(5)(q31),del(11)(q21) + ace, del(12)(q15),del(5)(q14)
S4	46,XY	5/94 (5.3)	3/94 (3.2)	. . .	2/26 (7.7)	del(3)(p14) + ace,del(14)(q21) + ace
S5	46,XX	13/84 (15.5)	40/84 (47.6)	tr(7;13)(q21;q13)	5/28 (17.9)	del(1)(q24) + ace,del(7)(q21),t(5;17)(q22;q25), 19q + , + dup(1)(q23-q32)
S6	46,XX	6/100 (6.0)	4/100 (4.0)	. . .	8/17 (47.1)	del(2)(p23-q23),del(5)(q22), + ace, del (4)(q28),t(1;6)(p34;q36) + t (7;12)(q36;q15), del(1)(p34),del(12)(q15) + ace + ace + ace
S7	46,XY	7/84 (8.3)	14/84 (16.7)	. . .	1/39 (2.6)	del(1)(q42) + ace
S8	46,XX	9/110 (8.2)	18/110 (16.4)	. . .	6/24 (25.0)	del(6)(p21.3-q16) + ace,ace(6)(q16q24), ace(6q24-qter),del(13)(q21) + ace, del(14)(q22),del(7q11.1) + ace
S9	46,XY	13/62 (21.0)	18/62 (29.0)	tr(5;7)(q13;p22)	1/20 (5.0)	del(12)(q24.1) + ace
S10	46,XX	10/100 (10.0)	17/100 (17.0)	tr(1;2)(p36;q32) + 2ace,cx(2;6;7) (p16;p21;q21)	2/26 (7.7)	del(16)(q12.1),t(7;16)(p15;p12)
S11	46,XX	24/108 (22.2)	45/108 (41.7)	tr(1;2)(?;q?) tr(13;18)(q12;q11.2) tr(2;17)(p12;q23)	4/33 (12.1)	del(7)(q21) + ace,del(8)(q11.1) + ace, tdic(7;10)(q34;q23),del(3)(q21) + ace
S12	46,XY	5/99 (5.1)	4/99 (4.0)	. . .	5/32 (15.6)	t(10;12)(q23;p13),tdic(6;10)(q24;p12), del(15)(q15),del(17)(q23) + ace,tdic(?)
S13	46,XY	21/115 (18.3)	40/115 (34.8)	tr(17;19)(q25;p13.2)	19/54 (35.2)	del(2)(p22) + ace,del(2)(q33) + ace, del(3)(p14) + ace,del(3)(p21) + ace + ace, mar,mar, del(7)(p13),del(7)(p11.2) + ace, del(7)(q11.2),del(9)(q22),del(9)(q31) + ace, del(10)(q11.2) + ace,t(?;16)(?; p12), der(4)(4;17)(p11;p11.1), der(17)(4;17)(q11.1,q11.1) + ace, del(17(p12), + mar, + mar, + mar, ace(17)(q21qter),mar,mar,mar,del(3)(p14) + ace, del(7)(p11.2),del(10)(q11.2) + ace, del(2)(p22) + ace,del(17)(p12), + ace, del(10)(q11.2), + ace, del(9)q(31) + ace,del(2)p(22) + ace, del(2)q(33) + ace,del(17)p(12)
S14	46,XY	7/102 (6.9)	4/102 (3.9)	. . .	3/36 (8.3)	del(3)(p11),del(11)(p13),del(18)(p11.3)
S15	46,XY	18/112 (16.1)	23/112 (20.5)	tr(12;17)(q13;q21)	5/26 (19.2)	del(18)(p11.2),del(18)(q21.3) + mar, inv(7)(p15.1;q31.2) inv(7)(p15.1;q31.2),del(18)(q21.3) + mar
S16	46,XX	10/106 (9.4)	36/106 (34.0)	. . .	1/39 (2.6)	47,XX, + mar
S17	46,XX	7/99 (7.1)	4/99 (4.0)	. . .	5/36 (13.9)	del(5)(p11.1),del(6)(q14), ace(15q22-qter), + mar,tdic(9;9)(q22;q32)
S18	46,XX	14/123 (11.4)	16/123 (13.0)	. . .	0/30 (0)	. . .
S19	46,XY	14/109 (12.8)	18/109 (16.5)	tr(?)	6/31 (19.4)	del(1)(q32) + ace,del(10)(q24) + ace,mar, mar,ace,tdic(3;?)
S20	46,XY	10/65 (15.4)	11/65 (16.9)	tr(13;?)(q21;?)	2/26 (7.7)	del(11)(q13.19), + mar
S21	46,XY	8/109 (7.3)	12/109 (11.0)	. . .	0/29 (0)	. . .
S22	46,XY	18/120 (15.0)	16/120 (13.3)	. . .	4/33 (12.1)	del(4)(p15.2),del(6)(q21),del(21)(q21), t(1;11)(p36;q11.1)
S23	46,XY	7/100 (7.0)	10/100 (10.0)	. . .	0/28 (0)	. . .
S24	46,XY	3/97 (3.1)	3/97 (3.1)	. . .	0/26 (0)	. . .
S25	46,XY	8/83 (9.6)	8/83 (9.6)	. . .	3/20 (15.0)	del(20)(p11.2) + ace,der(17p +), + ace

*Commas indicate the beginning of a new metaphase.

banding in structural abnormalities and in chromosomal lesions in the smoker group and of the 259 breakpoints in the control group was not uniform (FIGURE 2). With the exception of chromosome 22 in the smoker group and of chromosomes 21, 22, and Y in the control group, all other chromosomes were involved in structural abnormalities or in chromosomal lesions. To determine the possible existence of an association between the breakpoints found (at the 400-band resolution level) and those chromosome bands containing fragile sites, the data on fragile sites accepted by the Committee on Human Gene Mapping 11 were used.[19] The *t* test showed a preferential location of breakpoints in chromosome bands containing fragile sites, both in smokers and in controls ($P<.001$ and $P=.002$, respectively).

The fragile site multinomial method was used to identify those chromosome bands that significantly expressed breakpoints in the 2 groups. In both groups, the number of breaks required to consider a band to be nonrandomly affected was 4 or more. The results in the control group indicated that 12 bands were nonrandomly affected: 2q35, 7p15, 10q22, 11q13, and 14q24 (4 times each); 1p34, 1p22, 4q31, 6q21, and 12q13 (5 times each); and 1q32 and 17q21 (6 times each). In the smokers group, 30 bands were nonrandomly affected: 1p34, 1q42, 2p13, 2p16, 2p23, 2q21, 3q21, 5q15, 6q22, 7p15, 15q24, 16q22, 16q23, and 17q23 (4 times each); 1q23, 2p21, 4q31, 6p21, 11q13, and 12q15 (5 times each); 1p36, 1q11.2, 1q32, 3p14, 7q11.2, 7q32, and 9q22 (6 times each); 11q23 (9 times, but only in smokers) (FIGURE 3); 5q31 (10 times); and 17q21 (13 times) (TABLE 6).

To identify the bands with a greater propensity to break in smokers relative to controls, the differences in the number of breaks for the bands listed above were calculated as described in the "Methods" section. The mean of these differences was 0.72 (SD, 1.78), with –3 and 9 the most negative and positive values. Applying the criterion of 3 SDs of the computed differences from their mean value as a classifying

Table 5. Cytogenetic Results in Amniocytes From Fetuses Carried by Nonsmoking Controls

Control	Karyotype of Fetus	No./Total (%)		Triradials and Quadriradials	Structural Abnormalities, No./Total (%)	Types of Structural Abnormalities*
		Aberrant Metaphases	Cells With Gaps and Breaks			
C1	46,XX	5/95 (5.3)	5/95 (5.3)	. . .	0/28 (0)	. . .
C2	46,XX	7/100 (7.0)	7/100 (7.0)	. . .	2/27 (7.4)	del(1)(q11.2) + ace,del(12)(q11) + ace
C3	46,XY	3/91 (3.3)	4/91 (4.4)	. . .	0/27 (0)	. . .
C4	46,XY	8/102 (7.8)	6/102 (5.9)	. . .	2/31 (6.5)	del(11)(q11),tdic(5;10)(q23;q21) + ace(10)(q21-qter)
C5	46,XY	5/90 (5.6)	7/90 (7.8)	. . .	0/27 (0)	. . .
C6	46,XX	5/98 (5.1)	6/98 (6.1)	. . .	1/32 (3.1)	del(7)(p21) + ace
C7	46,XX	8/94 (8.5)	13/94 (13.8)	. . .	1/29 (3.4)	t(1;7)(p22;p15)
C8	46,XY	9/95 (9.5)	22/95 (23.2)	. . .	1/24 (4.2)	t(7;11)(q31;q24)
C9	46,XX	6/92 (6.5)	10/92 (10.9)	. . .	1/26 (3.8)	del(11)(p11.1) + ace
C10	46,XX	16/92 (17.4)	27/92 (29.3)	. . .	2/29 (6.8)	del(3)(p14) + ace,del(11)(q14) + ace
C11	46,XY	8/100 (8.0)	16/100 (16.0)	. . .	0/30 (0)	. . .
C12	46,XX	7/123 (5.7)	6/123 (4.9)	. . .	1/33 (3.0)	del(10)(q22)
C13	46,XY	12/110 (10.9)	16/110 (14.5)	. . .	2/31 (6.5)	del(15)(q15) + ace,del(5)(p15.1)
C14	46,XX	8/107 (7.5)	7/107 (6.5)	. . .	1/28 (3.6)	del(9)(q21)
C15	46,XX	13/117 (11.1)	18/117 (15.4)	. . .	1/39 (2.6)	del(7)(p14)
C16	46,XY	12/107 (11.2)	13/107 (12.1)	. . .	1/33 (3.0)	t(X;1)(p22.2;q25)
C17	46,XX	8/122 (6.6)	8/122 (6.6)	. . .	2/34 (5.9)	der(14)t(14;17)(q32;q21),ace
C18	46,XX	5/110 (4.5)	4/110 (3.6)	. . .	1/29 (3.4)	del(11)(p12)
C19	46,XX	9/121 (7.4)	13/121 (10.7)	. . .	0/27 (0)	. . .
C20	46,XY	6/112 (5.4)	8/112 (7.1)	. . .	0/27 (0)	. . .
C21	46,XX	12/119 (10.1)	12/119 (10.1)	. . .	1/34 (2.9)	mar
C22	46,XX	9/109 (8.3)	9/109 (8.3)	. . .	0/27 (0)	. . .
C23	46,XX	3/106 (2.8)	2/106 (1.9)	. . .	1/25 (4.0)	del(10)(p11.1) + ace
C24	46,XY	15/105 (14.3)	14/105 (13.3)	qr(4;15)(q12;q15)	5/36 (13.9)	del(5)(q11.2) + ace,del(5)(q11.2) + ace, del(12)(q11) + ace,del(6)(q23) +ace, del(17)(q22) + ace
C25	46,XY	11/120 (9.2)	14/120 (11.7)	. . .	0/39 (0)	. . .

Abbreviations for Tables 4 and 5: ace, acentric fragment; cx, complex chromatid interchanges; del, deletion; der, derivative; dup, duplication; inv, inversion; mar, marker chromosome; p, short arm; q, long arm; qr, quadriradial; qter, terminal long arm; t, translocation; tdic, dicentrical translocation; tr, triradial. Ellipses indicate no triradials/quadriradials or structural abnormalities found.
*Commas indicate the beginning of a new metaphase.

distance, no bands with extreme negative values were detected, whereas 3 bands with extreme positive values were found: 17q21 (difference, 7), 5q31 (difference, 7), and 11q23 (difference, 9). The Fisher exact test and the nonparametric Wilcoxon rank-sum test reached statistical significance only for 11q23 ($P = .02$, both tests).

COMMENT

In this study, the main difficulty was to find heavy smokers (≥10 cigarettes/d for ≥10 years) who also smoked during pregnancy, and control women not exposed to tobacco at home or at work (total of 800 interviews required). Moreover, smokers and controls had to be free of exposure to other clastogenic agents and not consume alcohol, coffee, or tea. In the present study it was found that, under these conditions, fetuses from pregnant women who smoked had an increased frequency of chromosomal instability, evaluated by the presence of structural chromosomal abnormalities and chromosomal lesions.

Chromosomal instability and analyses of micronuclei in lymphocytes from peripheral blood have been successfully used as biomarkers of genotoxicity both for assessing DNA damage at the chromosomal level and for quantifying early adverse human health effects, in particular cancer.[20,21] Peripheral blood lymphocytes from heavy smokers (>30 cigarettes/d) or from children born to smokers show increases in structural chromosomal abnormalities, SCEs, micronuclei, or fragile-site expression.[7-10] In utero, only indirect data using chorionic villi have been published[11,12]; one study showed an increase in SCEs while the other found no increase in chromosomal lesions.

In our study, comparison of cytogenetic data between groups of smokers and controls showed important differences for the proportion of structural chromosomal abnormalities and to a lesser degree for the proportion of metaphases with chromosomal instability and for the proportion of chromosomal lesions. This propensity for a strong genotoxic effect in mothers who smoke (highest incidence of the most severe anomaly) is also observed for the chromosomal lesions, where the differences are more marked for breaks than for gaps (Table 3).

Taking into account the way in which both groups had to be completed, maternal age was by chance significantly higher in the smoker than in the control group. It is well known that maternal age is related to an increase in numerical chromosomal abnormalities

Figure 2. Distribution of Breakpoints in Amniocytes From Fetuses Carried by Mothers in the Smoker and Control Groups Displayed in the Idiogram (400-Band Resolution)

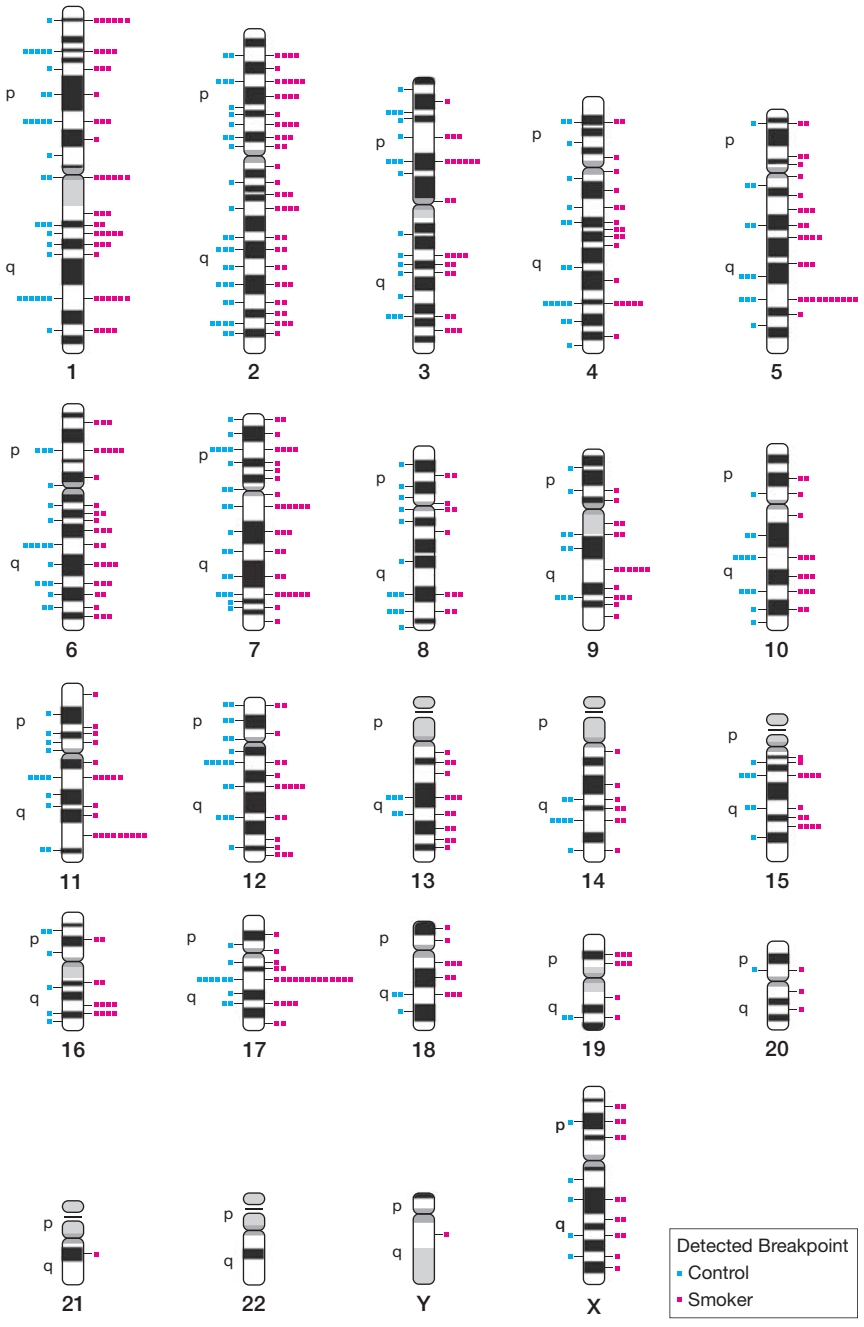
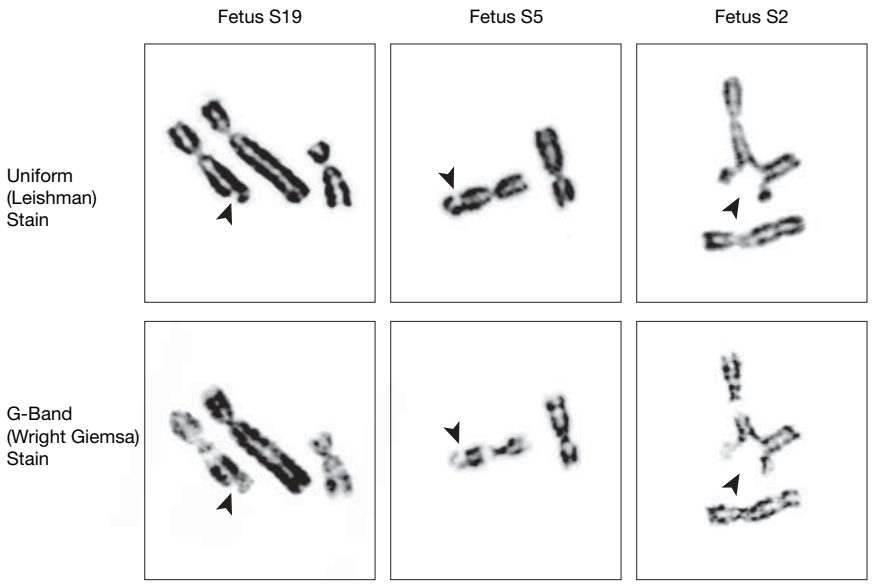


Figure 3. Partial Metaphases of Amniocytes From Fetuses Carried by Mothers Who Smoke, Showing Chromosomal Lesions on 11q23 Band



Arrowheads indicate the localization of gaps (fetuses S19 and S5) and a triradial (S2).

Table 6. Expression of Chromosomal Abnormalities on the Most Affected Chromosome Bands (5q31, 11q23, and 17q21) From Fetuses Carried by Mothers Who Smoke and From Nonsmoking Controls

	Participant No. (No. of Abnormalities)	
Band	Smokers	Controls
5q31	S2 (1), S3 (1), S5 (1), S8 (1), S11 (2), S13 (2), S16 (1), S19 (1)	C9 (1), C16 (1), C19 (1)
11q23	S2 (3), S5 (1), S14 (1), S15 (2), S19 (1), S22 (1)	
17q21	S3 (1), S5 (1), S13 (2), S15 (1), S17 (1), S18 (2), S19 (1), S21 (2), S22 (1), S25 (1)	C8 (2), C10 (1), C15 (1), C23 (1), C25 (1)

(especially trisomies and, among them, trisomy 21), but no study has related increasing maternal age to an increase in chromosomal lesions and structural abnormalities. Nevertheless, 2 GEE models were considered, either including or not including age as a covariate. In all the analyses performed, inclusion of age as a covariate led to an increase in the *P* value of the smoking factor relative to that in the reduced model. As a result, 2 of the analyses that showed significance in the reduced model, those for chromosomal instability ($P=.04$) and chromosomal lesions ($P=.045$), became nearly significant ($P=.05$) and marginally influential ($P=.10$), respectively, in the extended model. The third chromosomal anomaly studied, structural chromosomal abnormalities, remained significant in the extended model ($P=.01$). Finally, the analyses corresponding to a subset in which women who had become pregnant by in vitro fertilization or intracytoplasmic sperm injection were excluded showed similar significance values for the smoking factor in the extended model compared with the reduced model for all 3 chromosomal anomalies studied.

It is worth noting that the maternal age factor was not significant in any of the analyses performed, suggesting that the reduced model in which this factor was omitted could be more appropriate for the description of our data. Keeping a nonsignificant covariate in an extended model can be considered adequate when this factor belongs to the design configuration of the study or its association with the response variable is widely accepted in the research field. Neither of these circumstances applies in the present case. As indicated above, maternal age was an observational variable and it is numerical chromosomal abnormalities, not the anomalies studied in the present work, that are known to be associated with maternal age. Because of these reasons, a reduced model can be more suitable than the extended model including maternal age.

Our results show that fetuses exposed to tobacco smoke in utero have increased chromosomal instability in amniocytes, expressed as an increase of structural chromosomal abnormalities and chromosomal lesions, which is not influenced by maternal age. In the present study, no direct relationship between the level of genotoxic tobacco compounds and chromosomal instability has been demonstrated because the levels of tobacco-specific compounds (eg, cotinine) were not measured in amniotic fluid or maternal serum. However, the fact that several studies have described the presence of these compounds in the blood of fetuses from women who smoke[4-6] seems to support our findings, suggesting a possible genotoxic effect of smoking during pregnancy.

To determine if some chromosomal regions were especially affected by exposure of the fetus to tobacco, we localized the breakpoints implicated in chromosomal lesions and in structural abnormalities. An apparently nonrandom distribution of breakpoints and a coincidence with fragile-site bands in the smoker and control groups was observed. The preferential location of breakpoints in fragile-site bands in chromosomal preparations from chorionic villi has been previously described.[22,23] This coincidence has also been observed in lymphocyte chromosomes from cigarette smokers.[7,24] Recently, Stein et al[24] and Spitz et al[25] have stated that tobacco exposure increases chromosomal fragility due to an adaptation of DNA repair mechanisms to smoking, which in turn leads to an accumulation of genetic damage. It is worth noting that, according to these authors, this

ineffective repair is transient and reversible. Several data sets suggest that tobacco exposure induces in vivo fragile-site expression, which contributes to tumor formation.[26,27]

Our results show, in agreement with these studies, that tobacco exposure increases chromosomal instability due to late or incomplete DNA replication or to errors in repair mechanisms (inefficient response or poor inducible repair response). Both mechanisms may affect the integrity of chromosomal structure in these regions, leading to the appearance of structural chromosomal abnormalities, gaps, and breaks. Therefore, the chromosome breakpoints could produce deletions or disruptions of functional genes, producing developmental defects or genetic disorders, including cancer.

By comparing the breakpoint distribution in both groups using the fragile site multinomial method, 3 specific chromosome bands affected by exposure to tobacco have been detected: 5q31, 17q21, and, especially, 11q23. Breaks on 11q23, however, were only observed in smokers. Two of these bands, 5q31.1 and 11q23, correspond to regions where fragile sites FRA5C, FRA11B, and FRA11G are located. According to the Committee on Human Gene Therapy,[19] FRA5C and FRA11G are considered "fragile sites, aphidicolin-type, common" and FRA11B a "fragile site, folic acid-type, rare." In this sense, it should be noted that smokers have reduced concentrations of folic acid in serum,[28] a fact that could explain the high incidence of breakpoints at 11q23.

It is worthwhile to note that chromosome breaks at 3p14.2, where the most common fragile site (FRA3B) is located, were only found in mothers who smoke (6 times). Although in the present study this site was not among the 3 breakpoints most expressed in amniocytes from fetuses of mothers who smoke (more than 8 lesions each), this finding is consistent with that of a previous study[24] in which FRA3B expression is directly correlated with cigarette smoking.

It has been suggested that the increase of chromosomal lesions and structural abnormalities or the very existence of an increased chromosomal instability resulting from the genotoxic effect of tobacco could be indicative of an increased cancer risk and that fragile sites could be responsible for the chromosomal instability observed in cancer cells.[27] Moreover, an increase of chromosomal instability is associated with an increase in the risk of cancer, especially childhood malignancies.[29]

For the last 30 years, consumption of tobacco by parents has been related to leukemia in infancy.[2,30] It is known that a high proportion of infants (40%-60%), children (18%), and adults (3%-7%) with leukemia have molecular rearrangements in chromosome band 11q23, but these rearrangements are not always detectable by cytogenetic analysis.[31-34] According to some authors,[31-35] there is strong evidence that 11q23 rearrangements occur in utero. These findings show the importance of the involvement of band 11q23 in events leading to leukemogenesis in infants. The other 2 bands most affected by tobacco in our study (5q31 and 17q21), although not affected in statistically significant proportions, are also involved in childhood leukemia.[34]

In conclusion, maternal smoking of 10 or more cigarettes per day for 10 or more years, including during pregnancy, is associated with increased chromosomal instability in amniocytes. Band 11q23, which seems to be especially sensitive to compounds contained in tobacco, is known to be involved in leukemogenesis. This band contains the genes *ATM* (cell prolymphocytic leukemia), *PLZF* (leukemia acute, promyelocytic; *PLZF/RARA* type), and *MLL* (leukemia, myeloid/lymphoid, or mixed lineage). Thus, the transplacental exposure to tobacco could be associated with an increased risk of pediatric hematopoietic malignancies. Epidemiologic studies will be needed to determine whether the offspring of parents who smoke have an increased lifetime risk of cancer.

Author Contributions: Dr Egozcue had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Study concept and design; analysis and interpretation of data: de la Chica, Giraldo, Egozcue, Fuster.
Acquisition of data: Ribas.
Drafting of the manuscript: de la Chica, Ribas, Giraldo, Egozcue, Fuster.
Critical revision of the manuscript for important intellectual content; study supervision: Giraldo, Egozcue, Fuster.
Statistical analysis: Giraldo.
Obtained funding: Fuster.
Administrative, technical, or material support: de la Chica, Ribas.

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The greatest test of courage on earth is to bear defeat without losing heart.
—Robert G. Ingersoll (1833-1899)

