SHORT REPORT

Skewed X chromosome inactivation and early-onset breast cancer

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Background: Skewed X chromosome inactivation may be more common in women with epithelial ovarian cancer and early-onset breast cancer. We tested this hypothesis in a group of 235 breast cancer patients and 253 controls (mean age 45.8 years) from a larger population based case control study.

Methods: We measured X chromosome inactivation with the *AR* gene assay in lymphocyte DNA digested with the methylation specific enzyme *Hpa*II. We judged skewness using an adjusted measure (relative to the undigested sample) with a cut point of 75%, and an unadjusted measure where skewed was defined as >90% of the signal from one allele in the *Hpa*II digested sample.

Results: There were no significant differences in any of the skewing measures between cases and controls. Using the adjusted skewing measure among pre-menopausal subjects under the age of 50, 14% of cases versus 11% of controls were skewed, OR = 1.2, 95% CI 0.6 to 2.3; using the unadjusted measure, OR = 0.9, 95% CI 0.4 to 2.0.

Conclusions: While we cannot rule out a subtle difference of approximately twofold or less, we have failed to find a significant difference in the prevalence of skewed X chromosome inactivation in younger women with breast cancer compared to controls.

Random X chromosome inactivation in female embryos results in roughly equal expression of transcripts from maternally and paternally derived X chromosomes.[1] Skewed (or non-random) X chromosome inactivation is present if most, or all, of the transcripts are derived from only one of the parental X chromosomes.[2 3] Two case control studies have suggested that skewed X chromosome inactivation, measured in lymphocytes, is more common in ovarian cancer and early-onset breast cancer patients than in controls.[4 5] Skewed X chromosome inactivation may play a role in carcinogenesis if, for example, the preferentially expressed chromosome contains mutations or polymorphisms in cancer related genes. In addition, the recent observation of the involvement of the BRCA1 protein in normal X chromosome inactivation further highlights the possible involvement of X chromosome inactivation in breast cancer.[6] We studied whether skewed X chromosome inactivation was more common in early-onset breast cancer patients from a population based case control study conducted in Poland.

METHODS

Subjects

Subjects were drawn from a population based case control study of 2386 patients with breast cancer and 2503 controls aged 19–74 years living in Warsaw and Łódź, Poland. This study was approved by Institutional Review Boards at the Cancer Center and M. Sklodowska-Curie Institute of Oncology, Warsaw and Nofer Institute of Occupational Medicine, Łódź in Poland and at the US National Cancer Institute, National Institutes of Health, USA. Subjects were newly diagnosed with pathologically confirmed in situ or invasive breast cancer between February 1, 2000 and January 31, 2003, and controls were randomly selected from population lists, frequency matched on city of residence and age in 5 year categories (Garcia-Closas, submitted for publication and Fergenbaum *et al*[7]). Response rates for the personal interview were 79% for patients and 69% for controls, and of those interviewed, 84% of patients and 92% of controls gave a blood sample. Blood samples were collected on average 38 days after diagnosis. DNA was extracted from buffy coats using the automated PUREGENE DNA Purification Kit (Gentra Systems, Minneapolis, MN). The target subject group for the present analysis, selected in August 2003, included those who reported that they were still having menstrual periods (that is, pre-menopausal subjects) for whom DNA was already available. Only those patients with invasive breast cancer who were not known to have had chemotherapy before sample collection were included. We identified 644 subjects meeting these criteria.

Because laboratory analyses were begun before data collection was completed, 59 subjects analysed for skewed X chromosome inactivation were later determined to be postmenopausal or had received chemotherapy prior to blood collection. Subject recruitment has since been completed, and the 280 premenopausal women with invasive breast cancer and no history of chemotherapy included in this analysis represent 67% of all potentially eligible subjects. Sixty five subjects were homozygous at the androgen receptor (*AR*) locus (see Laboratory methods below), 79 samples did not amplify reliably, and two subjects gave discordant results, leaving 235 cases and 253 controls analysed for skewed X chromosome inactivation.

Laboratory methods

A trinucleotide repeat in the *AR* gene on the X chromosome is highly polymorphic, methylation at two CpG sites near the repeat correlates with silencing, and this locus has been used in numerous studies of skewed X chromosome inactivation.[2 8–10] Each DNA sample is amplified twice: once after it is digested with a methylation specific restriction enzyme (*Hpa*II) and once after it is digested with an ''irrelevant'' enzyme (*Rsa*I) that does not cut within the *AR* amplicon (herein referred to as ''undigested''). Primer sequences were as previously described[9] except that the forward primer was labelled with 6-FAM and products were analysed on an ABI 3730 sequencer and with GeneMapper software (Applied Biosystems, Foster City, CA). Because it resulted in peaks with heights that were more similar in the undigested state,

Table 1 Characteristics of study subjects

Characteristic	Eligible subjects (n = 644)				Subjects analysed for skewing (n = 488)			
	Patients (n = 314)		Controls (n = 330)		Patients (n = 235)		Controls (n = 253)	
	n	%	n	%	n	%	n	%
Study site								
Warsaw	184	59%	215	65%	143	61%	157	62%
Łódź	130	41%	115	35%	92	39%	96	38%
Age								
25–35	8	3%	10	3%	7	3%	7	3%
35–39	26	8%	26	8%	22	9%	21	8%
40–44	71	23%	78	24%	58	25%	61	24%
45–49	127	40%	148	45%	92	39%	111	44%
50–54	64	20%	54	16%	45	19%	39	15%
55–65	18	6%	14	4%	11	5%	14	6%
Age at menarche								
⩽12	109	35%	76	23%	86	37%	57	23%
13	70	23%	78	24%	46	20%	63	25%
14	80	26%	104	32%	61	27%	82	33%
15	26	8%	31	10%	16	7%	22	9%
⩾16	24	8%	36	11%	21	9%	24	10%
Missing	5		5		5		5	
No. of full term births								
Nulliparous	39	12%	33	10%	34	14%	25	10%
1	106	34%	113	34%	79	34%	91	36%
2	134	43%	138	42%	97	41%	102	40%
⩾3	35	11%	46	14%	25	11%	35	14%
Missing	0		0		0		0	
Age at first full term birth of parous women								
Nulliparous	39		33		34		25	
<20	29	11%	30	10%	23	11%	25	11%
20–24	135	49%	156	53%	97	48%	119	52%
25–29	80	29%	74	25%	58	29%	58	25%
⩾30	31	11%	37	12%	23	11%	26	11%
Missing	0		0		0		0	
Menopausal status								
Pre-menopausal	292	93%	305	92%	220	94%	233	92%
Postmenopausal	22	7%	25	8%	15	6%	20	8%
Missing	0		0		0		0	
Family history of breast cancer in first degree relatives								
No	286	91%	312	95%	213	91%	240	95%
Yes	28	9%	18	5%	22	9%	13	5%
Missing	0		0		0		0	

Tumour characteristics	Eligible cases (n = 314)		Cases analysed for skewing (n = 235)	
	n	%	n	%
Histological subtype				
Ductal	205	65%	155	66%
Lobular	43	14%	33	14%
Other	66	21%	47	20%
Lymph node status				
Negative	189	61%	140	61%
1–3	78	25%	61	26%
4+	42	14%	30	13%
Missing	5		4	
Received chemotherapy before blood collection				
No	302	96%	227	97%
Yes	12	4%	8	3%
Oestrogen receptor				
Negative	88	34%	61	32%
Positive	172	66%	130	68%
Missing	54		44	
Progesterone receptor				
Negative	168	65%	126	66%
Positive	92	35%	65	34%
Missing	54		44	

we used Herculase (Stratagene, La Jolla, CA) rather than Taq Gold (Applied Biosystems, Foster City, CA) polymerase enzymes. We calculated the proportion of the signal derived from the shorter (allele 1) peak as {allele 1 peak height/(allele 1 peak height+allele 2 peak height)}. When the difference in length between the two alleles was one repeat unit (3 bp), we adjusted the peak height by 27% because of the influence of stutter bands.

Statistical analyses

Skewing is not an all-or-nothing phenomenon but continuous measures of skewing were not normally distributed, nor were various transformations of the data. We therefore evaluated skewing with two non-parametric measures, the Wilcoxon two sample rank sum test (Z statistic with continuity correction of 0.5, two tailed) and the median two sample test (Z statistic, two tailed). We also categorised

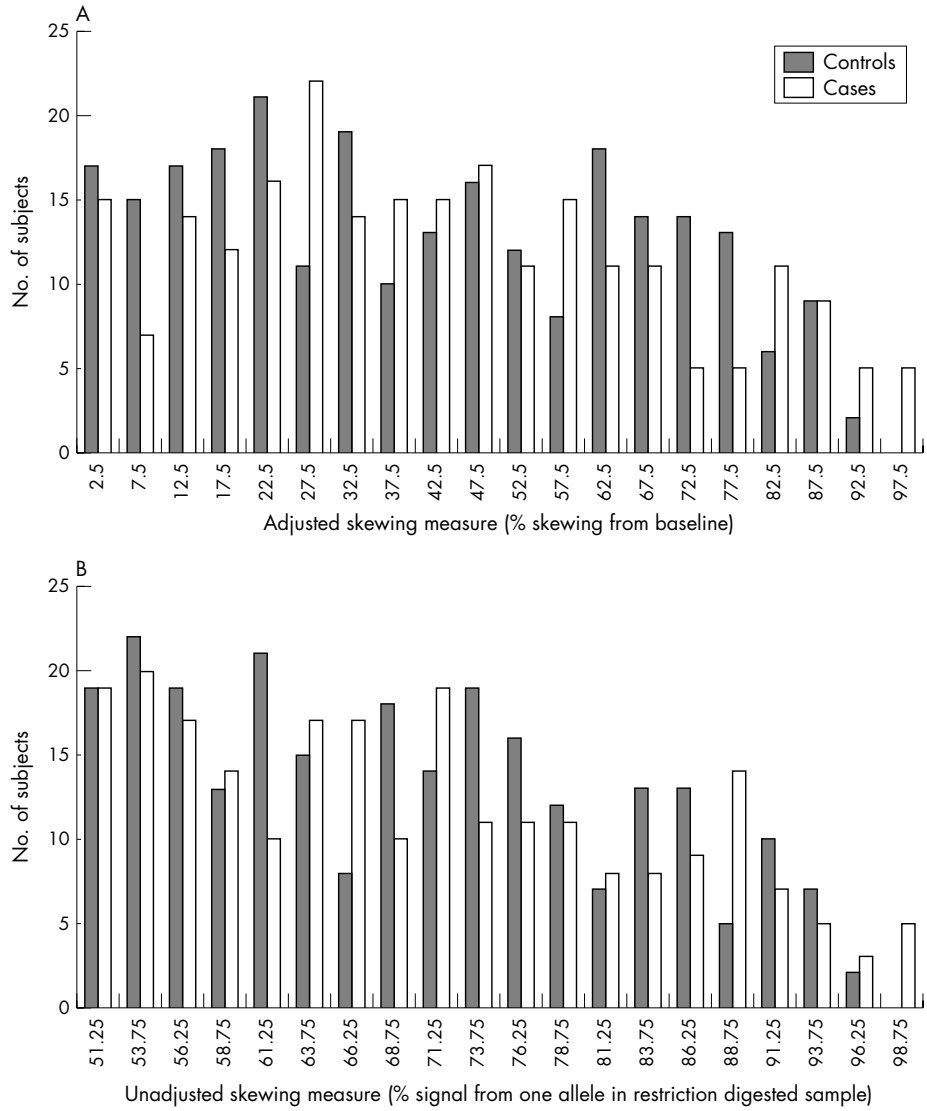

Figure 1 (A) Distribution of adjusted skewing measure according to case control status. Frequency histogram showing the number of subjects in each 5 percentage point interval of the adjusted skewing measure (% skewed from baseline, range 0% to 100%). Controls are indicated by black bars; breast cancer patients by white bars. (B) Distribution of unadjusted skewing measure according to case control status. Frequency histogram showing the number of subjects in each 2.5 percentage point interval of the adjusted skewing measure (% signal from one allele in restriction enzyme digested sample, range 50% to 100%). Controls are indicated by black bars; breast cancer patients by white bars.

subjects as skewed using both adjusted and unadjusted measures at various cut points. Because in the undigested state the peak heights were not always equal, we devised an adjusted skewing measure by using the allele 1 peak proportion in the undigested sample as a baseline. For example, if the allele 1 peak proportion was 40% in the undigested sample, the allele 1 proportion could increase 60 percentage points (to 100%) or decrease 40 (to 0%). If the allele 1 peak proportion was 70% in the digested sample, the % skewing was calculated as 50% (that is, 30 percentage points of a potential 60), and if it was 10% in the digested sample, the % skewing was 75% (30/40). Samples were attempted at least three times and 195 samples were scored twice. For subjects with two readings, the % skewed was averaged between the two runs. Those with $<$50%, 50–74%, and $\geqslant$75% skewing were categorised as normal, partially skewed, and skewed. In keeping with several prior publications, we also used an unadjusted measure by considering only the peak heights in the *Hpa*II restriction enzyme digested sample, categorising samples as skewed if more than 90% of the signal proportion was from one allele. Odds ratios were calculated using logistic regression, adjusting for age, age at menarche, number of full term births, age at first full term birth, menopausal status (when appropriate), and first degree family history of breast cancer. For the 195 subjects with two adjusted skewing measurements, the intraclass correlation coefficient[11] was calculated with the macro INTRACC (http://ftp.sas.com/techsup/download/stat/intracc.html). All statistical analyses were performed with SAS 9.1 (SAS Institute, Cary, NC).

RESULTS

The subset of subjects and controls available for this analysis, performed before subject recruitment was completed, were well matched to the entire case group with regard to area of residence and age (the two matching variables for the study overall), and those subjects who could be analysed for skewed X chromosome inactivation (because they were heterozygous for the *AR* polymorphism) did not differ from the entire case group (table 1). Among 195 subjects with two

Table 2 Association between breast cancer case control status and measures of skewed X chromosome inactivation

	All subjects (n = 488)							Pre- menopausal, no chemo, <50 years (n = 360)†						
	Controls (n = 253)		Cases (n = 235)					Controls (n = 194)		Cases (n = 166)				
	n	%	n	%	OR‡	95% CI	p value	n	%	n	%	OR‡	95% CI	p value
Adjusted % skewing*														
Normal (<50)	157	62.1	147	62.6	Reference (1.0)			126	64.9	106	63.9	Reference (1.0)		
Partial (50–74)	66	26.1	53	22.6				46	23.7	37	22.3			
Skewed (>75)	30	11.9	35	14.9	1.2	(0.7 to 2.1)		22	11.3	23	13.9	1.2	(0.6 to 2.3)	
Non-parametric tests														
Wilcoxon rank sum							0.4							0.3
Median							0.8							0.3
Unadjusted skewing measure§														
Normal	230	90.9	211	89.8	Reference (1.0)			177	91.2	152	91.6	Reference (1.0)		
Skewed (>0.9, <0.1)	23	9.1	24	10.2	1.1	(0.6 to 2.0)		17	8.8	14	8.4	0.9	(0.4 to 2.0)	
Non-parametric tests														
Wilcoxon rank sum							0.7							0.7
Median							0.9							0.8

*Absolute value of the percent change in proportion of signal from the shorter allele (allele 1) in the restriction enzyme digested sample compared to the undigested proportion.
†Includes only pre-menopausal women under the age of 50 with no history of chemotherapy before blood collection.
‡Odds ratio for showing skewed X chromosome inactivation (compared to the combined group of normal and partial), adjusted for age, age at menarche, number of full term births, age at first full term birth, menopausal status (all subjects group only), and first degree family history of breast cancer.
§Proportion of signal from the tallest allele in the restriction enzyme digested sample only.

Table 3 Characteristics of two prior studies of X chromosome inactivation and cancer and the current study

Setting	Subject characteristics/ number informative	Age range	Family history	Assay*	Results
US university hospital obstetrics/gynaecology department (University of Iowa)†	Invasive epithelial ovarian cancer (n = 174), unrelated controls (n = 45), matching not specified	Not fully specified, mean ~57	Unknown selection for family history, 11 *BRCA1* mutation carriers	*AR*, ^{32}P labelled DNA analysed with acrylamide gels; skewed if visually inspected peak ratio in digested samples ⩾3	53% of cases skewed, 33% of controls skewed, OR = 2.6
Two Norwegian hospitals‡	Consecutive series of breast cancer patients dx 1984–1994 (n = 40 diagnosed at ages 27–45), controls mostly blood donors (n = 90, age 19–45)	27–90, median 60	Unselected for family history	*AR*, fluor labelled DNA analysed on ABI 373; skewed if 90% or more of signal was from one allele in digested sample	13% of cases skewed, 1% of controls skewed, OR = 13
Subset of a population based study in two cities in Poland (current study)	Rapidly ascertained patients (n = 166, diagnosed at age <50), randomly selected population controls (n = 194, age <50), matched on residence and age	25–65, 92% between 35 and 54	Unselected for family history	*AR*, fluor labelled DNA analysed on ABI 3730; skewed if 75% or more of signal was from one allele in digested sample, adjusted to signal in undigested sample	14% of cases skewed, 11% of controls skewed, OR = 1.2

*All three studies used the same androgen receptor (*AR*) locus,[9] but with slightly different platforms/conditions, as outlined in the table.
†Buller *et al*.[4]
‡Kristiansen *et al*.[5]

laboratory skewing determinations, the intraclass correlation coefficient was 0.93. The distribution of the adjusted and unadjusted skewing measures among all cases and controls is shown in fig 1. Non-parametric analyses did not reveal a significant difference in skewing between cases and controls (table 2). Using the % skewed measure (adjusted for the allele proportion in the undigested sample), p values were 0.4 and 0.8 for the Wilcoxon rank sum and median test statistics in the entire sample and 0.3 for both statistics in pre-menopausal subjects below the age of 50. Using the unadjusted measure of the proportion of signal from one allele in the restriction enzyme digested sample only, p values were 0.7 and 0.9 for the Wilcoxon rank sum and median test statistics in the entire sample, and 0.7 and 0.8 in pre-menopausal subjects below the age of 50.

Logistic regression analyses, comparing the proportion skewed (calculated with both the adjusted and unadjusted measures) between cases and controls, similarly did not show a significant difference (table 2). Among all analysable subjects, slightly more cases (14.9%) than controls (11.9%) were skewed using the adjusted measure with a cut point of 75%, but this difference was not statistically significant (adjusted OR = 1.2, 95% CI 0.7 to 2.1). Nor was there significant difference among pre-menopausal women below the age of 50 (adjusted OR = 1.2, 95% CI 0.6 to 2.3). The unadjusted measure of skewing was not significantly different either (9.1% in all cases *v* 10.2% in controls; adjusted OR = 1.1, 95% CI 0.6 to 2.0). Among pre-menopausal women below the age of 50, the proportion skewed using the unadjusted measure was nearly identical. Analyses stratified by

family history of breast cancer were conflicting between the adjusted and unadjusted skewing measures: the adjusted skewing measure was somewhat lower in family history positive subjects compared to those without a family history (9% *v* 15% in cases and 8% *v* 12% in controls), while for the unadjusted measure, family history positive cases and controls had higher skewing (14% *v* 10% in cases and 15% *v* 9% in controls). These analyses, however, were based on only 22 and 13 family history positive subjects in the cases and controls, respectively.

DISCUSSION

We have studied younger women with breast cancer from a large population based case control study and have not found a significant difference in the proportion showing skewed X chromosome inactivation using an androgen receptor (*AR*) gene assay in lymphocyte DNA. This is in contrast to a report of a higher prevalence of skewed X chromosome inactivation in a study of early-onset breast cancer from Sweden,[5] and to the initial report of higher prevalences in women with epithelial ovarian cancer.[4] Our study is the largest to date, and while we cannot rule out more subtle differences between patients and controls, the upper confidence intervals on our odds ratios were all 2.3 or less.

The hypothesis that skewed X chromosome inactivation may play a role in breast and ovarian cancer aetiology was initially generated from a study of 174 informative women with epithelial ovarian cancer and 45 controls without ovarian cancer (table 3).[4] That study used the same *AR* PCR amplicon and defined ''skewed'' as those where the ratio of peak heights for the two alleles (determined visually) was $\geqslant 3$ in the *Hha*I restriction enzyme digested lymphocyte DNA sample. Cases were much more likely to be skewed (53%) compared to controls (33%) (unadjusted OR = 2.6). They also noted that nine of 11 *BRCA1* mutation carriers among the cases were skewed. This observation heightened our interest in the subsequent basic research finding that the BRCA1 protein is involved in normal embryonic X chromosome inactivation.[6] A small study of breast cancer patients and controls from Sweden also found higher rates of skewed X chromosome inactivation in their patients.[5] They defined ''skewed'' as those demonstrating 90% or more of the signal from one allele in the *Hpa*II restriction enzyme digested lymphocyte DNA samples using the *AR* assay. In contrast to older patients, 13% of 40 subjects diagnosed at the age of 45 or younger were skewed compared to 1% of 95 controls (OR = 13).

There is no clear standard for categorising a sample as showing skewed (non-random) X chromosome inactivation. A commonly used assay is that used in this study in which the highly polymorphic trinucleotide repeat within exon 1 of the *AR* gene is amplified in a PCR reaction using DNA that is pre-digested with *Hpa*II (or *Hha*I). These enzymes do not cut DNA if there is methylation of the cytosines in the two recognition sequences within this PCR amplicon. If X chromosome inactivation is random, the peak heights representing the two chromosomes (the two alleles) will be nearly equal (owing to equal methylation and therefore equal restriction digestion of two alleles). Departures from equal peak heights indicate varying degrees of inactivation. Ideally this departure from equal allele peak heights would be treated as a continuous variable and subjected to analysis of variance, but numerous methods of quantifying this skewing, along with numeric transformations, were not normally distributed. This requires one to use potentially less powerful non-parametric tests and to arbitrarily determine cut points at which samples are categorised as skewed or not. Particularly because the relative peak heights of the two alleles were not equal even in assays of the DNA processed with mock restriction digests (undigested DNA), we devised the adjusted skewing measure before linking the laboratory and epidemiological variables. To facilitate comparison with most prior publications, we also categorised samples using only the information in the restriction digested peak heights.

Upon inspection of the frequency histograms for both adjusted and unadjusted skewing measures (fig 1), one gets a hint of more cases at the very extreme of the tail. None of our analyses using numerous cut points for the various measures calculated before inspection of these figures, however, showed a significant difference between cases and controls, including for the smaller group of women age 45 and younger (data not shown). This is not to say that there are no possible cut points that might result in a post hoc comparison with a p value less than 0.05. Non-parametric tests similarly did not confirm a significant difference. We designed this study to have at least 80% power to detect a twofold increase in risk, assuming 15% of controls would show skewing ($\alpha = 0.05$). Using our adjusted skewing measure, approximately 12% of controls showed skewed X chromosome inactivation, which would have reduced our power very slightly. However, despite numerous outcome measures and statistical tests, we did not observe a significant difference in the rates of skewed X chromosome inactivation, and the upper confidence intervals around our odds ratios were generally 2.0 to 2.3. Our data suggest that skewed X chromosome inactivation, as measured in peripheral lymphocytes using the *AR* gene assay, is not more common in younger women with breast cancer compared to controls.

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ELECTRONIC-DATABASE INFORMATION

Information on the macro INTRACC is available from http://ftp.sas.com/techsup/download/stat/intracc.html.

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REFERENCES

1 **Avner P**, Heard E. X-chromosome inactivation: counting, choice and initiation. *Nat Rev Genet* 2001;**2**(1):59–67.
2 **Busque L**, Gilliland DG. X-inactivation analysis in the 1990s: promise and potential problems. *Leukemia* 1998;**12**(2):128–35.
3 **Brown CJ**. Skewed X-chromosome inactivation: cause or consequence? *J Natl Cancer Inst* 1999;**91**(4):304–5.
4 **Buller RE**, Sood AK, Lallas T, Buekers T, Skilling JS. Association between nonrandom X-chromosome inactivation and *BRCA1* mutation in germline DNA of patients with ovarian cancer. *J Natl Cancer Inst* 1999;**91**(4):339–46.
5 **Kristiansen M**, Langerod A, Knudsen GP, Weber BL, Borresen-Dale AL, Orstavik KH. High frequency of skewed X inactivation in young breast cancer patients. *J Med Genet* 2002;**39**(1):30–3.
6 **Ganesan S**, Silver DP, Greenberg RA, Avni D, Drapkin R, Miron A, Mok SC, Randrianarison V, Brodie S, Salstrom J, Rasmussen TP, Klimke A, Marrese C, Marahrens Y, Deng CX, Feunteun J, Livingston DM. BRCA1 supports XIST RNA concentration on the inactive X chromosome. *Cell* 2002;**111**(3):393–405.
7 **Fergenbaum JH**, Garcia-Closas M, Hewitt SM, Lissowska J, Sakoda LC, Sherman ME. Loss of antigenicity in stored sections of breast cancer tissue microarrays. *Cancer Epidemiol Biomarkers Prev* 2004;**13**(4):667–72.
8 **Allen RC**, Zoghbi HY, Moseley AB, Rosenblatt HM, Belmont JW. Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. *Am J Hum Genet* 1992;**51**(6):1229–39.
9 **Giovannucci E**, Stampfer MJ, Krithivas K, Brown M, Dahl D, Brufsky A, Talcott J, Hennekens CH, Kantoff PW. The CAG repeat within the androgen receptor gene and its relationship to prostate cancer. *Proc Natl Acad Sci U S A* 1997;**94**(7):3320–3.
10 **Beever CL**, Stephenson MD, Penaherrera MS, Jiang RH, Kalousek DK, Hayden M, Field L, Brown CJ, Robinson WP. Skewed X-chromosome inactivation is associated with trisomy in women ascertained on the basis of recurrent spontaneous abortion or chromosomally abnormal pregnancies. *Am J Hum Genet* 2003;**72**(2):399–407.
11 **Shrout PE**, Fleiss JL. Intraclass correlations – uses in assessing rater reliability. *Psychol Bull* 1979;**66**(2):420–8.