ORIGINAL ARTICLE

High frequency of skewed X inactivation in young breast cancer patients

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Introduction: Patients with invasive ovarian cancer were recently shown to have a higher frequency of skewed X chromosome inactivation in peripheral blood cells compared to patients with borderline cancer and controls. In this study, we analysed the X inactivation pattern in peripheral blood from 216 breast cancer patients.

Methods: X inactivation analysis was performed using *Hpa*II predigestion of DNA followed by PCR of the highly polymorphic CAG repeat of the androgen receptor gene (*AR*), which amplifies the undigested inactive X chromosome only. The X inactivation pattern was classified as skewed when 90% or more of the cells preferentially used one X chromosome.

Results: Young breast cancer patients (27-45 years) had a higher frequency of skewed X inactivation than young controls (13 and 1%, respectively) (p=0.009), whereas no difference was found for middle aged and older patients compared to controls of a similar age.

Conclusions: A germline mutation in an X linked tumour suppressor gene may give a proliferative advantage to cells with this mutation on the active X chromosome, thus causing skewed X inactivation and an increased risk for developing cancer. Another possible explanation could be that females with a constitutionally skewed X inactivation pattern are more susceptible to develop breast cancer because of an X linked low penetrance susceptibility allele that is affected by the inactivation pattern.

In female mammalian cells, one of the two X chromosomes is inactivated in early embryonic life. Females are therefore mosaics for two cell types, cells with the paternal X chromosome as the active X chromosome, and cells with the maternal X chromosome as the active X chromosome. Most females have a 50:50 distribution of the two cell types. A deviation from this distribution may occur, giving a skewed X inactivation. Skewed X inactivation may be the result of chance, or genetic factors involved in the X inactivation process,[1 2] or a selection process. Female carriers of some X linked disorders, such as Wiskott-Aldrich syndrome, Lesch-Nyhan syndrome, Barth syndrome, and some of the immunodeficiency syndromes, have skewed X inactivation, presumably as a result of a post-inactivation selection against cells with the mutated gene on the active X chromosome.[3–6] Older females have an increased frequency of skewed X inactivation in peripheral blood cells, most probably also as a result of a selection process.[7–9]

An association between skewed X inactivation and ovarian cancer was recently reported.[10] A higher frequency of a skewed X inactivation pattern was found in patients with invasive cancer compared to patients with borderline cancer and healthy controls, indicating that skewed X inactivation may be associated with a predisposing factor for the development of invasive ovarian cancer. An increased frequency of skewed X inactivation was also found in patients who were carriers of germline *BRCA1* mutations.[10] It was therefore of interest to study if there is an association between skewed X inactivation and breast cancer. In this report we describe the results of X inactivation analysis of 216 breast cancer patients.

The androgen receptor (AR) functions as a transcriptional activator of androgen regulated genes. The highly polymorphic CAG trinucleotide repeat in the first exon of the *AR* gene encodes a polyglutamine tract in the N-terminal transactivation domain of AR. Shorter CAG repeats are associated with higher transcriptional activity of *AR*.[11] Both short and long *AR* CAG repeats have been associated with breast cancer development.[12 13] We therefore analysed the *AR* CAG repeat size for any association between breast cancer and repeat size.

SUBJECTS

Breast cancer patients

The breast cancer cases were part of a consecutive series of blood and tumour samples that had been collected after informed consent at the Norwegian Radium Hospital and Ullevål Hospital, respectively, from 1984 to 1994. Median age at diagnosis was 60 years, range 27-90 years.

Controls

Since there is a tendency for X inactivation to be more skewed with advancing age, it was necessary to have controls of different age groups. The control populations were 144 Norwegian blood donors aged 20-40 years, 138 blood donors aged 19-65 years (median 40 years), and 202 females aged 73-93 years (median 77 years). In addition, we had a control population of 91 females aged 55-72 (median 65 years), who were part of a routine mammography screening programme where blood samples were collected after two negative screenings (table 1).

METHODS

DNA isolation

DNA was extracted from peripheral blood cells and from tumour tissue according to standard procedures, using the automated phenol extraction method (Nucleic Acid Extractor 340A, Applied Biosystems).

X chromosome inactivation analysis

The X inactivation pattern was determined by PCR analysis of a polymorphic CAG repeat in the first exon of the *AR* gene.[14] Methylation of *Hpa*II sites in close proximity to this repeat correlates with X chromosome inactivation. After digestion with the methylation sensitive enzyme *Hpa*II, a PCR product is obtained from the inactive X chromosome only. PCR products

Abbreviations: AR, androgen receptor

Table 1 X chromosome inactivation in breast cancer patients and controls

	Age range (median)	Subjects with skewed X inactivation No (%)	Total number	p value
Patients	27–40 (38)	4 (22)	18	0.003
Controls	20–40	3 (2)	144	
Patients	27–45 (41)	5 (13)	40	0.009
Controls	19–45 (33)	1 (1)	95	
Patients	27–65 (51)	10 (7)	136	0.005
Controls	19–65 (40)	1 (0.7)	138	
Patients	55–72 (63)	7 (8)	91	0.35
Controls	55–72 (65)	4 (4)	91	
Patients	73–90 (78)	6 (14)	43	0.27
Controls	73–93 (77)	43 (21)	202	

from undigested and digested DNA were separated on an ABI 373 automated sequencer and analysed by GeneScan software (Applied Biosystems) (fig 1).

X inactivation pattern was recorded as the relative amount of the PCR product of the smallest allele, where 0 indicates a pattern where the smallest allele is the predominating active X chromosome and 100 indicates a pattern where the largest allele is the predominating active X chromosome. The X inactivation pattern was classified as skewed when 90% or more of the cells preferentially used one X chromosome.

Statistical methods

The Pearson chi-square test was used for testing categorical variables. The Fisher two tailed exact test was used where appropriate; p values less than 0.05 were taken as statistical significance.

RESULTS

X inactivation in breast cancer patients

Two hundred and sixteen patients were heterozygous for the CAG repeat in the *AR* gene and therefore informative in the X inactivation assay. Since females aged 60 years or older have a much higher frequency of skewed X inactivation than younger females,[7 8] the frequency of patients with a skewed X inactivation was determined separately for the various age groups (table 1).

A skewed pattern was found in the younger patients (≤48 years) and in the elderly patients (≥64 years) only. The frequency of skewed X inactivation in the patients was significantly higher than in the controls, both when the youngest patients (27-40 years) were compared to the young control group (20-40 years) (22% and 2%, respectively) (p=0.003), and when the patients aged 27-65 years were compared to the blood donor control group aged 19-65 years (7% and 0.7%, respectively) (p=0.005) (table 1). When presumably premenopausal patients only (≤45 years) were compared to blood donors of the same age group, the frequencies were also different (13% and 1%, respectively) (p=0.009). Information on the absence of breast cancer after mammography screening was available in the controls aged 55-72 only. When this group was compared with patients of the same age group, a difference in the frequency of skewed X inactivation was also found (4% and 8%, respectively), but the difference was not significant (p=0.35). In the elderly patients (73-90 years), the frequency of skewed X inactivation was lower than in a population of elderly controls (73-93 years) (14% and 21%, respectively), but the difference was not significant (p=0.27) (table 1).

Tumour tissue from four young patients (31-44 years) only was available and informative for X inactivation analysis. The X inactivation in tumour tissue was compared to the X inactivation in peripheral blood cells. Two patients with random X inactivation in peripheral blood cells had a similar pattern in tumour tissue. Two patients had a skewed X inactivation in blood cells (≥90%) and a skewing of 80% in tumour cells with a preference for the same cell line as observed in blood.

AR CAG repeat length

There was no difference in CAG repeat size between patients and controls. The median repeat length for the shorter allele was 20 (range 6-26) for cases and 20 (range 12-27) for controls. The median repeat length for the longer allele was 24 (range 19-31) for cases and 23 (range 18-31) for controls. Two cut off points, six and 30 repeats, were determined to evaluate if CAG repeat extremes were associated with breast cancer. A repeat size of 30 or more was more frequent in the young

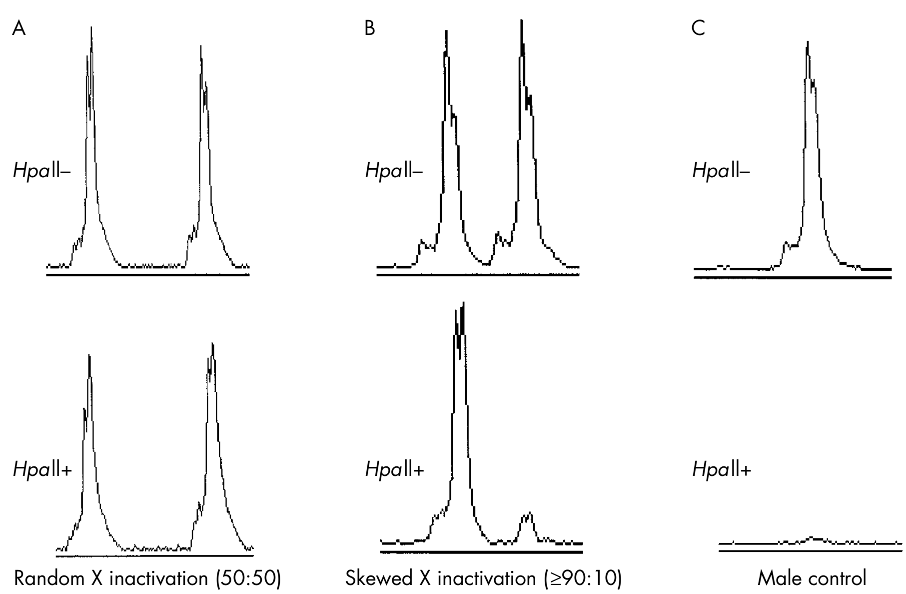

Figure 1 X inactivation in breast cancer patients. *Hpa*II– indicates no predigestion and *Hpa*II+ indicates predigestion with *Hpa*II. (A) Random X inactivation pattern. (B) Skewed X inactivation pattern. (C) Male control. A PCR product is obtained for the inactive X chromosomes only. Note lack of PCR product after *Hpa*II digestion of male control.

patient group (7.5%) compared to the control group (2%), but the difference was not significant (p=0.12).

The possibility existed that there was a preferential inactivation of the shorter and most functional allele, or a preferential inactivation of the longer and less functional allele in the breast cancer patients. No such preferential inactivation was found. In the patients, the shorter allele was the preferentially active allele in 49%, whereas in the total control population, the shorter allele was the preferentially active allele in 52%.

DISCUSSION

In this study, we found a higher frequency of skewed X inactivation in young breast cancer patients than in control females. Middle aged and old patients did not have a higher frequency than their respective controls.

Buller *et al*[10] found that patients with invasive ovarian cancer had an increased frequency of skewed X inactivation compared to patients with borderline tumours or controls. Furthermore, they found a skewed X inactivation in nine of the 11 patients where a *BRCA1* mutation was identified. Our results are in agreement with their findings. However, in our study, an increase in skewing was found for the younger patients only (table 1). In the report by Buller *et al*[10] age was not considered.

The authors suggested that an X linked gene is a risk factor for the development of ovarian cancer and discussed two models, both involving a mutation in an X linked tumour suppressor gene. In the first model, skewed X inactivation is a chance occurrence, inactivating the X chromosome with the normal copy of the gene, and thus leading to inactivation of both copies. In the second model, cells with the mutated copy of the gene on the active X chromosome have a proliferative advantage, thus leading to a skewed X inactivation.

The lack of an increased frequency of skewed X inactivation in older breast cancer patients could imply that a proportion of those females who are born with skewed X inactivation develop cancer at younger ages, and are therefore not included in the older patient group. This supports the hypothesis that skewed X inactivation, or a factor associated with it, is a risk factor for the development of early onset breast cancer.[10]

The possibility existed that the skewed X inactivation in the patients is related to chemotherapy, since chemotherapy may cause neutropenia and lymphopenia. Information on the timing of blood sampling in relation to therapy was not available for the patients in our study. However, no difference in X inactivation pattern was found between females who had received chemotherapy and controls in a study by Gale *et al*.[15] Furthermore, in the report by Buller *et al*,[10] several breast cancer patients who had been given chemotherapy were examined for X inactivation pattern more than a year after chemotherapy, with no change in X inactivation pattern. A significant effect of chemotherapy on X inactivation pattern would also be expected to affect the X inactivation in middle aged and elderly patients, where no increase in X inactivation pattern was found.

It would be of interest to study the relationship between the X inactivation pattern in blood and tumour tissue. In this study, we found skewing in the same direction in blood and tumour cells in the only two young skewed cancer patients where tumour tissue was available, but no conclusions may be drawn from this limited material.

The role of the *AR* gene in breast cancer development is poorly understood.[16 17] Both short and long *AR* CAG repeats have been associated with breast cancer development.[12 13] Yu *et al*[12] found that shorter CAG repeat length was associated with more aggressive forms of breast cancer. Spurdle *et al*[18] did not find any difference in mean *AR* CAG repeat length between females who developed breast cancer before the age of 40 and controls. In this study, we found no evidence for a different distribution of alleles between young breast cancer patients and controls. However, we found a tendency for extreme CAG repeats of 30 or more to be more frequent in the young breast cancer patients. This finding is in agreement with Rebbeck *et al*,[13] who found that longer *AR* CAG repeats were associated with an increased risk of developing breast cancer at an early age in *BRCA1* mutation carriers.

We found a higher incidence of skewed X inactivation in young patients with breast cancer. These results need to be verified in a larger sample. Since patients with familial breast cancer have an earlier onset of breast cancer than the sporadic cases,[19] it will be of interest to see whether the increased frequency of skewed X inactivation is limited to patients with *BRCA1* or *BRCA2* mutations. It will also be of interest to examine the relationship between various histological characteristics of the tumour and X inactivation pattern.

Skewed X inactivation can lead to the expression of recessive traits in females who are heterozygous for X linked disorders, either as the result of the chance occurrence of skewed X inactivation or as the result of a selection process. An increase in skewing of X inactivation in females with breast cancer may therefore be an indication of an effect of X linked genes. It would also be of interest to study the X inactivation pattern in females with other cancers.

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