Skewed X chromosome inactivation of blood cells is associated with early development of lung cancer in females

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Abstract. The skewed X chromosome inactivation (SXCI) was found mainly in adult females. It has been linked to development of ovarian and breast cancers. The present study aimed to describe the relationship between SXCI and development of lung cancer in females. DNA was isolated from blood cells from patients with primary lung cancer (n=148) and reference subjects (n=289). The androgen receptor (AR) gene exon 1 was amplified, with its products from different alleles resolved on denaturing polyacrylamide gels and visualized by silver staining. The corrected ratio (CR) between products from AR alleles after and before *Hpa*II pretreatment was calculated. Occurrence of SXCI was detected in both the patients and reference subjects at similar frequency. However, the phenomenon was more frequent in the patients below 40 years compared to the corresponding reference group, either taking CR ≥3 (25 and 5.8%, respectively; P=0.048) or CR ≥10 as the criterion of SXCI (16.7 and 0.8%, respectively; P=0.022). A higher frequency of SXCI was also found in the patients below 50 years compared to that for the corresponding reference group when CR ≥10 adopted as the criterion (7.9 and 1.2%, respectively; P=0.046). The cancer patients with SXCI were more than 10 years younger in average age than those without SXCI. SXCI of blood cells is associated with early development of lung cancer in females. The X chromosomal inactivation assay, therefore, may be used to screen for females predisposed to malig-nancies including lung cancer.

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Introduction

Random X chromosome inactivation, also known as lyonization, results in the transcriptional silencing of almost all genes located in one of the two X chromosomes of diploid female cells (1). It occurs during the interphase early in embryogenesis when one X chromosome is randomly inactivated by extensive methylation and condensation to form the sex chromatin body. The remaining, uncondensed one becomes the active X chromosome and is inherited by all daughter cells through subsequent mitotic division. Because this process is random, adult female tissues are cellular mosaics, wherein half of the cells contain an active maternal X chromosome (Xm) and the other half contain an active paternal X chromosome (Xp) (2,3).

Theoretically, the ratio of the paternal inactive X-linked allele to maternal one should be 1:1, and any significant deviation from the ratio is termed as skewed X chromosome inactivation (SXCI) (4,5). Lyonization ratio (inactive Xp/Xm) of a large population of females follows a Gaussian distribution in which SXCI is a statistically rare event (6). Though a few mechanisms have been proposed for SXCI (7), little is known about its significance. In 1999, Buller *et al* showed that SXCI might be a predisposing factor for the invasive, but not border-line, ovarian cancer associated with BRCA1 mutations (8). Kristiansen *et al* found that young breast cancer patients had an increased frequency of SXCI in blood cells, suggesting SXCI might be associated with the early development of breast cancer (9,10). In the present study, we associated SXCI to the early development of lung cancer in females using an assay on peripheral blood cells.

Materials and methods

Samples and DNA extraction. Peripheral blood was taken from 148 female patients with lung cancer and 289 reference subjects. The patients were admitted to Tangdu Hospital, Fourth Medical Military University in Xi'an, P.R. China during the period from April 2003 to June 2005, with all of the cases diagnosed as a primary lung cancer through bronchofibero-scopy and/or pulmonary resection and pathological

examination. The patients were 19-78 (median, 61) years old at diagnosis, without a record of smoking or a family history with lung cancer. Among them, 39 patients had squamous cell carcinoma, 81 adenocarcinoma, 6 adenosquamous carcinoma, 18 small cell lung cancer (SCLC), 3 sarcomatoid carcinoma, and 1 had a carcinoid. Clinical stages were evaluated according to an international staging system (11), with 29, 36, 13, 16, 36 non-SCLC cases determined as stages I, II, IIIA, IIIB and IV, respectively, and with 19 and 2 SCLC cases as limited and extensive stages, respectively. The reference subjects were collected from April 2003 to June 2005. They were 10-80 (median, 60) years old, without a smoking history and without any personal or family record of a detectable cancer. Among them, 69 were staff members of Tangdu Hospital receiving a regular check-up, and the remaining were female patients admitted to Department of General Surgery of the hospital for surgical reasons other than a tumor. The examination was conducted with the consent of all the patients and reference individuals enrolled. The protocol was approved by the Medical Ethics Committee of The Fourth Military Medical University.

The blood samples were taken from the elbow vein before any therapy had been initiated, and were treated immediately with an anticoagulant containing sodium citrate (22 g/l) and sodium chloride (8.5 g/l) as described previously (12). Then the samples were stored at -70°C until use. Genomic DNA was isolated from the samples using a DNA extraction kit (Qiagen, Hilden, Germany). Concentration of DNA was determined by an ultraviolet spectrophotometer (Eppendorf, Hamburg, Germany).

Principles of X chromosome inactivation analysis. The assay was based on differential inactivation of X chromosomes of the female somatic tissues and the CAG short-tandem repeat (STR) polymorphism at the androgen receptor (AR) gene exon 1 (13). There are two *Hpa*II and two *Hha*I restriction sites at the locus 100 bp upstream to the CAG STR with a heterozygosity frequency around 90% (13,14). X chromosome inactivation is associated with methylation of these restriction sites. When these sites are methylated, as on the inactive X chromosome, the gene is not transcribed. When unmethylated, as on the active X chromosome or in males, the gene is transcribed (8). The digestion with methylation-sensitive endonucleases, followed by polymerase-chain reaction (PCR) with primers flanking these restriction sites and the highly polymorphic STR, can be used to distinguish between transcriptionally active and inactive X chromosome in heterozygous female subjects.

In the females with random X chromosome inactivation, the amplification products from both alleles should be equal, with a ratio of approximately 1:1. In the neoplastic tissues, most of which originate from single cell clones, the ratio is changed markedly compared to the surrounding tissues. The non-random X chromosome inactivation has been used in description of clonality status of lesions with undetermined nature (15-17). Meanwhile, remarkable deviation of the ration occurring in apparently non-neoplastic cell populations in females, such as blood cells, was defined SXCI (4,5).

Pretreatment of genomic DNA. DNA was digested by mixing sample DNA 10 μl (1 μg) with 0.5 μl of *Hpa*II (10 U/μl;

Promega, Madison, WI, USA), 2 μl of 10 mol/l reaction buffer, 0.2 μl of 10 g/l bovine serum albumin and 7.3 μl of deionized water. The final volume was 20 μl. The mixtures were then incubated at 37°C for 4 h. The reaction was terminated by incubation at room temperature for 30 min as suggested by the manufacturer.

Amplification of the AR gene exon 1. Nested PCR was conducted as previously described (15,16). Two pairs of PCR primers (13) were used: AR1A, 5'-GAG GAG CTT TCC AGA ATC TG-3'; AR1B, 5'-CAT GGG CTT GGG GAG A-3'; AR2A, 5'-TCC AGA ATC TGT TCC AGA GC-3'; AR2B, 5'-TGG GGA GAA CCA TCC TCA CC-3'. The first-round reaction was in a 50-μl volume containing DNA template 5 μl, 10 mmol/l deoxynucleotide triphosphate 4 μl, 50 mmol/l MgCl$_2$ 1.5 μl, 20 pmol/l primers AR1A and AR1B, 1 μl each, and 0.25 unit of Taq DNA polymerase (Takara Biotechnology Co., Ltd., Dalian, P.R. China). Twenty-five cycles were performed under the condition of 97°C for 40 sec, 56°C for 50 sec, and 72°C for 1 min, with a final extension at 72°C for 15 min. The amplification products were diluted at 1:10 and subjected to second-round reaction using the primer pair AR2A/AR2B and the same conditions as for the first one.

Electrophoresis. The amplification efficacy was demonstrated through electrophoresis on 2% agarose gels. The amplification products, 4 μl for each and mixed with the same volume of loading buffer (1 g/l xylene cyanole, 1 g/l bromophenol blue, in formamide), were then loaded onto the 10% polyacrylamide gel containing 8 mol/l urea, resolved through electrophoresis with the Mini-VE system (Amersham Biosciences Corp., San Francisco, CA, USA) under a voltage of 80 v for 8 h, and visualized by silver staining as described previously (15). For the samples whose allelic difference at the CAG STR was small (one or two repeats), the long gel (26 cm long and 0.75 mm thick) was used for the resolution with the SE660 system (Amersham). The results were recorded and intensities of the products from both alleles were analyzed using an image-analyzing system (LabWorks 3.0, UVP, Cambridge, UK).

Quality control and SXCI assessment. As PCR is prone to contamination, special attention was paid to avoid the problem. A negative, water-blank control was always included in each batch of PCR reactions. If the negative control was shown to be positive, the reaction was repeated for the whole batch. The reaction fidelity of *Hpa*II pretreatment was guaranteed by parallel negative controls with the enzyme omitted from the reaction mixture. In addition, the assays were carried out twice in independent series.

To rule out the possibility of incomplete digestion that may result in underestimation of SXCI, the pretreatment conditions were optimized for the assay. A DNA sample from mononuclear blood cells of a healthy man was treated with *Hpa*II for 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 h separately, with the residual intact DNA detected by nested PCR as described above. If the digestion is sufficient, subsequent PCR reaction will yield no amplification signal, since male somatic cells have only one X chromosome and the chromosome is active.
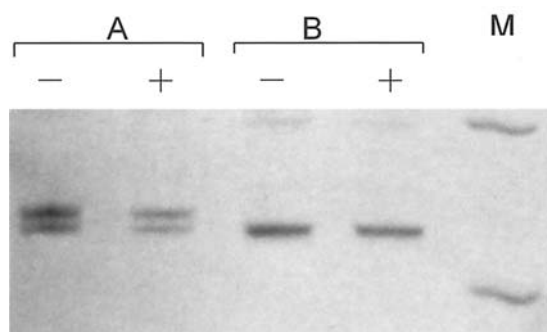
Figure 1. Representative data of amplification products of of the AR gene exon 1, with (A) and without CAG STR polymorphism (B). +, with *Hpa*II pre-treatment; -, without *Hpa*II pretreatment; M, DNA marker, the upper and lower bands representing positions of 300 and 200 bp, respectively.

Figure 2. Efficacy of digestion with the methylation-sensitive restriction enzyme *Hpa*II. A DNA sample from mononuclear blood cells of a healthy male were incubated with the enzyme for the time periods from 1.0 to 4.0 h, with the enzyme replaced by deionized water in the negative control (-). M, DNA marker with bands at positions of 500, 400, 300 and 200 bp from top to bottom, respectively.

In order to avoid interference of possible preferential amplification of one of the alleles, we used the corrected ratio (CR) to evaluate the X chromosome inactivation pattern by comparing the allelic difference of a sample before and after the *Hpa*II pretreatment. CR was derived by dividing the ratio of the upper-band intensity to the lower-band intensity of the sample after digestion by that of the same sample before the pretreatment. If CR was <1, the reciprocal value was considered. In this study, CR ≥3:1 was regarded as SXCI, corresponding to expression of the same allele in about 75% of the cells examined. As proposed by previous authors (6,18-20), we also used CR ≥10 as the more stringent criteria for SXCI.

Statistical analysis. Statistical computations were performed using an SPSS statistical package (Version 13.0; SPSS Inc., Chicago, IL, USA) for Windows. The χ^2 test was used for evaluating categorical variables. Student's t-test was performed for the comparison of continuous variables. All of the P-values are two-tailed and set at 0.05 for significance.
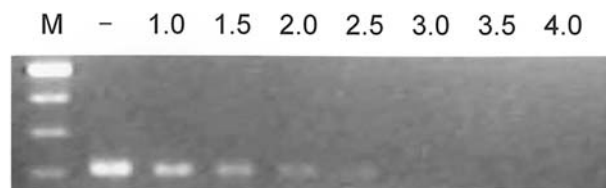
Results

Amplification for AR gene exon 1 was successful in all of the samples from 148 patients with lung cancer and 289 reference subjects. Among the cancer patients, 127 (85.8%) were shown to be polymorphic at the CAG STR (Fig. 1), and thereby informative for X chromosomal inactivation analysis. The ages of the informative cases at diagnosis ranged from 19 to 78 years, with a median of 60 years. Among the reference subjects, 258 (89.3%) were polymorphic at the CAG STR. Their ages ranged from 10 to 80 years, with a median of 61 years. No significant difference was found between the polymorphism frequencies for the cancer patient and reference groups (P<0.05). With these data combined, the frequency of CAG STR polymorphism was 88.1% (385/437), being similar to that from other authors (6-10,13-17,18-20).

DNA samples from mononuclear blood cells of a healthy male were digested with *Hpa*II for the time periods from 1.0 to 4.0 h, and then subjected to the nested PCR. As shown in Fig. 2, no intact DNA was detected after 3 h of *Hpa*II pretreatment. The data presented clearly demonstrate the reliability of our digestion procedure (with *Hpa*II for 4 h).

Previous studies have shown that female aged 60 years or older have a much higher frequency of SXCI than younger

Table I. Frequencies of X-chromosomal inactivation skewing in lung cancer patients and references.

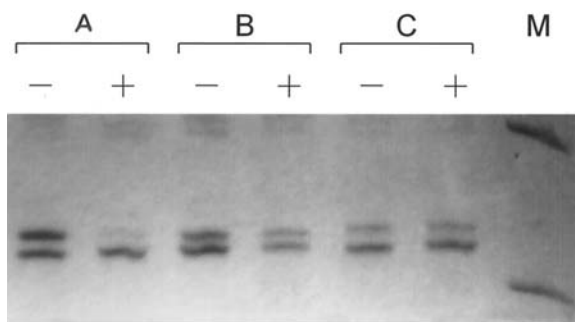| Groups	Age (years)	Numbers examined	Numbers with CR ≥3 (%)	P-value	Numbers with CR ≥10 (%)	P-value
Patients		12	3 (25.0)		2 (16.7)	
	<40			0.048		0.022
References		120	7 (5.8)		1 (0.8)	
Patients		38	6 (15.8)		3 (7.9)	
	<50			0.128		0.046
References		166	13 (7.8)		2 (1.2)	
Patients		89	6 (6.7)		4 (4.5)	
	≥50			0.064		0.488
References		92	11 (12.0)		5 (5.4)	
Patients	19-78	127	12 (9.4)		7 (5.5)	
				0.918		0.126
References	10-80	258	24 (9.3)		7 (2.7)	

Figure 3. Representative data of X chromosomal inactivation patterns in lung cancer patients, with SXCI in samples from patients A (CR=186, >10) and B (CR=4.4, >3) and without SXCI in the sample from patient C (CR=1.1, <3). M, DNA marker, with the upper and lower bands at positions of 300 and 200 bp, respectively.
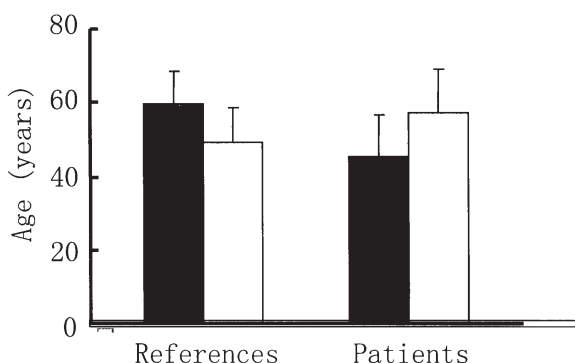

Figure 4. Average ages of the lung cancer patients and references with SXCI defined by CR ≥3 (filled bar) and without SXCI (empty bar). Their standard deviations denoted on each bar. The differences in both the reference and patient groups are statistically significant, with P-values <0.05 and <0.005, respectively.

females (6,9,20-23), and the age factor was taken into consideration in the present study. We also observed the association in the reference group, with SXCI frequencies of the references younger than 50 years and those aged up to 50 years, 7.8 and 12.8%, respectively. For this reason, the SXCI in patients and references was evaluated in various age groups (Table I).

Both CR ≥3 and CR ≥10 were used as criteria for SXCI to describe the X chromosomal inactivation skewing in the cancer patients (Fig. 3) and reference subjects. When CR ≥3 was adopted to determine SXCI, the incidences of the patient and reference groups were 9.4 and 9.3%, respectively; when CR ≥10 was adopted, the frequencies were 5.5 and 2.7%, respectively (Table I). The differences shown were not significant (P>0.05). However, the frequency in the patients below 40 years was significantly higher than that in the corresponding reference group, whether SXCI was defined as CR ≥3 (25 and 5.8%, P=0.048) or CR ≥10 (16.7 and 0.8%, P=0.022). For the patients and reference subjects younger than 50 years, the criteria adopted markedly affected evaluation of the X chromosomal inactivation skewing. When CR ≥10 adopted, SXCI incidences of these two groups were 7.9 and 1.2%, respectively, the former being significantly higher than the latter (P<0.05). When CR ≥3 adopted, the frequencies were 15.8 and 7.8%, respectively, and their

difference did not attain statistical significance (P=0.128). In patient and reference groups up to 50 years, the incidences of the excessive skewing as defined by CR≥3 and CR ≥10, were 6.7, 12% and 4.5, 5.4%, respectively. No significant difference was found between these two groups (P>0.05).

The average ages of the subjects with and without SXCI were determined for both groups (Fig. 4). Whether CR ≥3 or CR ≥10 was taken as the criterion, average age of the reference group with SXCI was more than 10 years older than that of the group without SXCI (P<0.05). In contrast, average age of lung cancer patients with SXCI was more than 10 years younger than that of those without SXCI (P<0.005 for CR ≥3; P<0.05 for CR ≥10).

The relationship between the SXCI and histological types or clinical stages of the cancer was evaluated. With the criterion of CR ≥3 adopted, SXCI frequencies in patients with squamous cell carcinoma, adenocarcinoma, small cell carcinoma and other types of lung cancer were 14.8% (4/27), 8.2% (6/73), 5.6% (1/18) and 10% (1/10), respectively. The frequencies for the non-SCLC cases of stages I, II, III and IV were 5.6% (1/18), 18.2% (4/22), 20.8% (5/24), and 2.9% (1/35), respectively. There was no significant difference among them (P>0.05).

Discussion

X chromosomal inactivation skewing was incidentally observed when Vogelstein *et al* established a clonality assay based on the AR gene polymorphism (4). The phenomenon was also noted in apparently healthy females. Its occurrence was then associated to age, with SXCI (CR ≥3) frequencies being 8.6, 16.4 and 38%, respectively, in neonates, young (28-32 years) and old females (>60 years) (6). In the present study, SXCI (CR ≥3) was also observed in the females without a detectable tumor, its frequencies being 7.8% in the references younger than 50 years and 12% in those aged up to 50 years. The average age of the reference subject with SXCI was 10 years older than those without SXCI. The data demonstrate that SXCI occurs in Chinese women with a lower frequency (9.3%) compared to that in the females from Western countries. Our study provides further evidence that SXCI does occur with aging.

Little is known about the clinical significance of SXCI. In 1999, Buller *et al* reported that patients with invasive ovarian cancer had an increased frequency of SXCI compared to those without a detectable fully malignant ovarian tumor (8), indicating that SXCI may be a predisposing factor for the development of invasive ovarian cancer. In a survey by Kristiansen *et al*, SXCI (CR ≥10) frequency was shown to be markedly increased in young patients (≤45 years) with breast cancer (13%) compared to that of the control group (1%), whereas the difference was not detected for the patients and reference subject >45 years (9). They also found a similar phenomenon in familial breast cancer patients without a detectable BRCA1 or BRCA2 mutation (10). In the present study, SXCI, as defined by CR ≥10, was observed more frequently in the female patients with lung cancer below 50 years (7.9%) compared to that in the reference group (1.2%). Additionally, whether CR ≥3 or CR ≥10 was adopted as the criterion for SXCI, the average age at diagnosis in cancer

patients with SXCI was 10 years younger than that without SXCI. The data prove that SXCI is a predisposing factor for the early development of lung cancer. Hence, SXCI may be a useful parameter to assess susceptibility of females to some malignancies including lung cancer.

While the assay proved to be convenient, several procedures were conducted to ensure its reliability and reproducibility. First, incomplete digestion of genomic DNA may result in underestimation of skewing after amplification of the DNA samples, which has been encountered in previous X chromosomal inactivation studies targeting the AR gene exon 1 (8,27-29). In order to rule out this possibility, a digestion test was conducted using *Hpa*II, showing elimination of all intact unmethylated DNA molecules after the pretreatment for 3 h. For the consideration of safety, we adopted 4 h of pretreatment in this study. Second, profound neutropenia and lymphocytopenia can occur after chemotherapy or radiotherapy. Theoretically, clonal expansions following the pancytopenic alterations may affect the SXCI patterns in blood cells. However, this is not true, as demonstrated in a few careful studies (5,8). In spite of this, we collected all the blood samples before chemotherapy or chemotherapy. In addition, the assay was repeated at least once to guarantee its reproducibility. In the present study, CR ≥3 was adopted as the criterion, as proposed by previous authors (6,18). In addition, we also took CR ≥10 as the more stringent criterion.

The pathogenesis of SXCI remains to be elucidated. In fact, SXCI in peripheral blood cells reflects imbalance of the cell population with an active Xm and that with an active Xp. The phenomenon also correlates with X chromosomal inactivation skewing in solid tissues (9,23), meaning that X chromosome may indeed harbor one or more negative growth regulation (tumor suppressor) genes, as postulated previously (8-10,24). We consider that, in some female individuals, there are germline mutations or the polymorphisms at these tumor suppressor genes on one X chromosome, either Xp or Xm. X chromosome inactivation during embryogenesis will result in loss of functions of one of the alleles. The cells with the methylated normal allele will lose all or most of the functions, whereas those with an active normal one will keep the functions. As a result, the former cell population obtains advantageous growth potential over the latter one. The underlying difference may become obvious gradually with the increase of surviving pressure in individuals affected by additional oncogenic factors, either intrinsic or environmental ones, resulting in the imbalance between the two populations and occurrence of SXCI.

In summary, SXCI in blood cells is associated with early development of lung cancer in females. The X chromosomal inactivation assay may be used to screen for females predisposed to some malignancies including lung cancer.

Acknowledgements

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