Technical Advance

A Novel, Essential Control for Clonality Analysis with Human Androgen Receptor Gene Polymerase Chain Reaction

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The most widely used technique for determining clonality based on X-chromosome inactivation is the human androgen receptor gene polymerase chain reaction (PCR). The reliability of this assay depends critically on the digestion of DNA before PCR with the methylation-sensitive restriction enzyme *HpaII*. We have developed a novel method for quantitatively monitoring the *HpaII* digestion in individual samples. Using real-time quantitative PCR we measured the efficiency of *HpaII* digestion by measuring the amplification of a gene that escapes X-chromosome inactivation (XE169) before and after digestion. This method was tested in blood samples from 30 individuals: 2 healthy donors and 28 patients with myelodysplastic syndrome. We found a lack of XE169 DNA reduction after digestion in the granulocytes of two myelodysplastic syndrome patients leading to a false polyclonal X-chromosome inactivation pattern. In all other samples a significant reduction of XE169 DNA was observed after *HpaII* digestion. The median reduction was 220-fold, ranging from a 9.0-fold to a 57,000-fold reduction. Also paraffin-embedded malignant tissue was investigated from two samples of patients with mantle cell lymphoma and two samples of patients with colon carcinoma. In three of these cases inefficient *HpaII* digestion led to inaccurate X-chromosome inactivation pattern ratios. We conclude that monitoring the efficiency of the *HpaII* digestion in a human androgen receptor gene PCR setting is both necessary and feasible. (*Am J Pathol* 2002, 161:807–812)

X-chromosome inactivation has been widely used to establish the clonal nature of malignancies. X chromosome

inactivation, also known as Lyonization, occurs early in embryogenesis and involves the random inactivation and methylation of either of the two X-chromosomes in all female cells.¹ Once established, the X-chromosome inactivation pattern (XCIP) of a particular cell will be transferred to all of the progeny of that cell. As a result a normal cell population of a healthy female usually shows a 50:50 ratio of inactivated paternal and inactivated maternal alleles. Because malignant cells are derived from a single precursor cell all tumor cells contain the same inactivated X-chromosome, resulting in a monoclonal XCIP.

One of the most important assets of clonality analysis is that monoclonality is always related to the first transforming mutation in the multistep pathogenesis of a tumor. This has been of particular importance in the investigation of premalignant lesions. For instance, clonality of suspected premalignant lesions has been confirmed in ovarian endometrial cysts,² Langerhans' cell histiocytosis,³ and atypical adenomatous hyperplasia of the lung.⁴ Clonality studies have also been used for the investigation of a common neoplastic origin of different cell types within a tumor^{5–7} or multiple tumor loci in the same patient.⁸ Analysis of clonality is of practical use when neoplastic and reactive conditions present with similar clinical symptoms, as in the vascular lesions of primary (clonal) and secondary (polyclonal) pulmonary hypertension⁹ and in idiopathic hypereosinophilic syndrome (clonal) and reactive eosinophilia (polyclonal).¹⁰ Also, clonal hematopoiesis has been shown to be predictive for the development of therapy-related myelodysplastic syndrome (MDS) or acute myeloid leukemia in non-Hodgkin's lymphoma patients after autologous bone marrow transplantation.¹¹

The most widely used method for XCIP determination utilizes a highly polymorphic trinucleotide repeat in the

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human androgen receptor gene (HUMARA). Approximately 90% of the female population is heterozygous for this polymorphism.¹² The combination of laser microdissection and the HUMARA polymerase chain reaction (PCR) assay¹³ allows clonality analysis in virtually all tissue samples of females. After PCR amplification of the HUMARA locus, the two alleles are present as two PCR products of different size. Discrimination between active and inactive alleles can be made by digestion of the DNA with a methylation-sensitive restriction enzyme, such as *HpaII*, before PCR. Because the inactivated X-chromosome is heavily methylated, it will not be digested, and only the inactivated allele will serve as a template for the PCR. Essential for this method is the digestion with *HpaII*, with incomplete digestion resulting in false polyclonality. Until now digestion of male DNA has been used for monitoring the efficiency of the *HpaII* digestion. However, in this way no information is obtained about the efficiency of digestion in individual samples.

We have developed a method that allows monitoring of *HpaII* digestion efficiency in individual samples. Several genes that escape X-inactivation have been identified on the X-chromosome. XE169 is one of the genes that escape X-inactivation^{14,15} and is located between Xp11.21 and Xp11.22 within a domain containing at least five other genes, all escaping X-inactivation.¹⁶ Digestion of *HpaII* sites in the XE169 gene is therefore not hindered by methylation and should result in the digestion of both alleles. We designed a real-time quantitative PCR spanning an *HpaII* site in the XE169 gene. We expressed the efficiency of *HpaII* digestion as the relative reduction of target DNA by comparing the amount of XE169 DNA before and after digestion. We investigated peripheral blood and bone marrow samples from MDS patients. MDS is a malignant bone marrow disease with frequent progression to acute myeloid leukemia. In MDS myeloid cells in the peripheral blood and bone marrow generally clonal,^{17,18} but polyclonal myeloid cells are seen in a number of cases.¹⁷⁻¹⁹ We investigated the efficiency of *HpaII* digestion in MDS patient samples with either a clonal or a polyclonal XCIP at diagnosis. Additionally, four paraffin-embedded tissue samples were investigated.

Materials and Methods

Sampling

Peripheral blood or bone marrow samples were obtained from 2 healthy blood donors (1 male, 1 female) and 28 female MDS patients after obtaining informed consent. MDS patients participated in the Clonal Remission after Intensive Antileukemic Therapy (CRIANT) study (study no. 06961) of the European Organization for Research and Treatment of Cancer in framework of the Biomed-2 program BMH4-96-0357. Of six patients both bone marrow and blood cells were investigated. Of 21 patients monocytes or T cells or both were also investigated. Of three patients one or more follow-up samples were investigated. The DNA of seven patients and one male healthy donor was digested twice with *HpaII* on two different

occasions. The DNA of the healthy female donor was used in five different digestions. In total, 77 different samples were used for *HpaII* digestion. In addition, four archival paraffin-embedded tissue samples were investigated; two samples from the lymph nodes of two patients with mantle cell lymphoma and two samples from colon carcinoma lesions of two different patients.

Selection of Granulocytes, Monocytes, and T Cells from Peripheral Blood and Bone Marrow

Preparation of cells was performed as described earlier.²⁰ All samples were separated in polymorphic nucleated cells (granulocytes) and mononuclear cells using Ficoll 1.077 density gradient centrifugation. Granulocytes (polymorphic nucleated cells) appeared to be more than 95% pure by flow cytometric analysis based on light-scattering properties. Mononuclear cells were washed and stained with mouse monoclonal antibodies for CD19 (phycoerythrin-conjugated) and CD3 (fluorescein isothiocyanate-conjugated) (Coulter Immunotech, Marseille, France). Cells were sorted using a Coulter Epics Elite flow cytometer (Beckman Coulter, Fullerton, CA). T cells were defined as CD3-positive, CD19-negative. Monocytes were defined as CD3- and CD19-negative, and having the appropriate scattering characteristics. After sorting, the cells were prepared for DNA isolation.

DNA Isolation, Digestion, and HUMARA PCR

DNA was isolated with the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN), both for the hematopoietic samples and the paraffin-embedded samples according to the protocols of the manufacturer. Subsequent HUMARA analysis was performed as described earlier.²⁰ Digestion of 0.5 µg of DNA was performed for at least 16 hours at 37°C in a reaction mixture of 25 µl, containing 5 U *DdeI* (Life Technologies, Gaithersburg, MD) and with or without 25 U of concentrated *HpaII* (New England Biolabs, Hitchin, UK) in 1× one-phor-all buffer PLUS (Amersham Pharmacia, Uppsala, Sweden). After digestion, 5 µl of this mixture was used for PCR amplification of the HUMARA locus. The PCR mixture contained 300 nmol/L of forward primer, 400 nmol/L of fluorescein isothiocyanate-labeled reverse primer, 6% dimethyl sulfoxide, 2.5 mmol/L dNTP's (Amersham Pharmacia), 1.5 mmol/L MgCl₂, 1× buffer II, and 1.25 U AmpliTaq Gold (Applied Biosystems, Foster City, CA) in a total reaction mixture of 50 µl. Primer sequences were: forward 5'-ccccaggcaccagagggc-3', reverse 5'-gagaaccatcctcacctgct-3'. PCR conditions were: 7.5 minutes at 95°C, followed by three rounds of 2.5 minutes at 95°C, 30 seconds at 62°C, 1 minute at 71°C, followed by 32 rounds of 45 seconds at 95°C, 30 seconds at 62°C, 1 minute at 72°C, followed by a single step of 10 minutes at 72°C.

Analysis of PCR Products

PCR products were analyzed as described before²⁰ by electrophoresis on agarose or acrylamide gels, and the

relative abundance of the alleles was quantified. Analysis was performed on 4% agarose E-gels (Invitrogen, Carlsbad, CA). If the relative abundance of either allele was more than 75% or if the difference in size was less than two repeats (6 bp), analyses were repeated on acrylamide gels. The relative abundance of alleles was quantified using a Gel Doc 1000 UV detection system and Multi Analyst software (Bio-Rad, Hercules, CA) for agarose gels. Acrylamide analysis was done using POP-4 acrylamide, genetic analyzer buffer and "310" capillaries (Applied Biosystems, Foster City, CA) in a P/ACE 5000 capillary electrophoresis system equipped with a LIF detector and an argon laser at 488 nm (Beckman Coulter, Fullerton, CA) or in a ABI PRISM 310 genetic analyzer (Applied Biosystems). All samples were analyzed at least in duplicate. The XCIP ratio was calculated by dividing the signal of the largest allele by the total signal of both alleles and multiplying with 100%. Samples were considered polyclonal if the XCIP ratio was between 75:25 and 25:75. More extreme XCIP ratios were considered to be clonal.

Real-Time XE169 PCR

Quantitative real-time PCR was performed with the ABI/PRISM 7700 sequence detection system for quantification with a fluorescent probe and the 5700 Sequence detection system for quantification with Sybr Green and melting curve analysis (Applied Biosystems). Primer sequences were: forward: 5'-gcttggtgtgacgcaacgta-3', reverse: 5'-gccttcgccaccacagttca-3'. The sequence of the TET-labeled probe was: 5'-aggacaccccgcggaaggatcc-3'. PCR conditions were as follows: 10 minutes at 95°C followed by 45 cycles of 15 seconds at 95°C and 1 minute at 57°C, with data collection in the last 30 seconds. For all PCRs 1.25 U of AmpliTaq Gold, 5 µl of 10× buffer A or 5 µl 10× Sybr Green buffer, 5 mmol/L MgCl₂ (all Applied Biosystems, Foster City, CA), and 250 mmol/L dNTPs (Pharmacia) were used in the reaction mixture. Primer concentrations were 900 nmol/L and the probe concentration was 200 nmol/L. All PCRs were performed in a total volume of 50 µl.

The reduction of XE169 copies was calculated by comparing the cycle threshold (Ct) value of two aliquots of the same sample; both were treated identically with the exception of the presence or absence of *HpaII* in the digestion mixture. This fold reduction was calculated by the formula: fold reduction = $2^{((Ct \text{ with } HpaII) - (Ct \text{ without } HpaII))}$. The efficiency of the PCR was determined from a serial dilution series of a digestion mixture of a DNA sample without *HpaII* in digestion mixture and was 1.93, expressed as the fold increase in fluorescence per PCR cycle, showing the validity of the method used for quantification. The correlation coefficient (R^2) for this dilution series was 0.996. PCRs for each sample were performed in duplicate.

Results

The *HpaII* digestion of the DNA from mononuclear cells of the peripheral blood of a healthy female donor was fol-

Figure 1. *HpaII* digestion time course of DNA from a healthy female donor. The digestion time in hours is plotted against the fold reduction of XE169 DNA after *HpaII* digestion. For each time point the amount of XE169 DNA was compared with real-time quantitative PCR between a reaction mixture with or without *HpaII* enzyme. The fold reduction after digestion reached a plateau between 4 and 24 hours. The DNA was isolated from the mononuclear cells of the peripheral blood.

lowed in time to monitor the efficiency of digestion. The amount of XE169 DNA was measured before and after 1, 2, 3, 4, or 24 hours of *HpaII* digestion (Figure 1). After 4 hours the digestion reached a plateau phase with an almost 500-fold reduction of XE169 DNA. All further samples were digested for at least 16 hours to ensure maximal digestion.

To test the *HpaII* digestion in individual DNA samples, 70 *HpaII* digestions were analyzed from 28 different female MDS samples and 1 healthy male donor sample. The importance of measuring *HpaII* performance in individual samples was shown in DNA samples from granulocytes of two patients. The reduction after *HpaII* digestion measured with the XE169 PCR was, respectively, 1.1- and 0.7-fold with Sybr Green detection and 1.9-fold for both samples with a fluorescent probe. These numbers were within the error margin of quantitative PCR, which cannot reliably detect differences less than two-fold. This implied that the *HpaII* digestion did not function in these samples. Using the HUMARA PCR, the same DNA samples showed a polyclonal XCIP ratio of 43:57 and 47:53, respectively. DNA from monocytes was also available of these patients. Analysis of *HpaII* digestion of this material yielded reductions of, respectively, 1400- and 39-fold using the XE-169 control assay and clonal XCIP ratios of 2:98 and 81:19 using the HUMARA PCR assay (Figure 2). Also the other 64 digestions of the DNA of 26 patients and the male donor showed a significant reduction of XE169 DNA (more than fivefold) after *HpaII* digestion, ranging from a 9.0-fold to a 57,000-fold reduction (median, 220-fold) (Figure 3).

Reproducibility of digestion and analysis was evaluated in eight samples that were digested in two independent experiments, with Sybr Green analysis in one and fluorescent probe analysis in the other experiment. The mean difference between two digestions was 2.3-fold, ranging from 1.2- to 3.7-fold indicating that the *HpaII* digestion is a reproducible process and that the Sybr Green and fluorescent probe analyses are comparable. The variation in the duplicate digestions was smaller than the variation in individual samples ($P < 0.0001$) indicating that the digestibility of a sample is more dependent

Figure 2. False polyclonality in the granulocytes of two MDS patients. The XCIP for four DNA samples of two MDS patients is represented by the relative intensity of the two alleles after HUMARA PCR. The two alleles were visible as peaks in the electropherogram of the PCR products, and are indicated with **A** and **B**. The relative intensities of the peaks are given as percentages. In both patients a polyclonal XCIP in the granulocytes coincided with a failure of *HpaII* digestion, whereas a clonal XCIP in the monocytes coincided with successful *HpaII* digestion. The success of *HpaII* digestion is expressed as the fold reduction of XE169 DNA measured with real-time quantitative PCR after digestion.

on the quality of the sample than on the performance of the *HpaII* enzyme.

DNA from archival, paraffin-embedded tissue was also tested for digestibility to check the feasibility of the described method for this kind of material. Four samples were tested from malignant tissue with different amounts

Figure 3. *HpaII* digestion is successful in the majority of samples. The quantity of XE169 in a reaction mixture without *HpaII* (in arbitrary units) is plotted against the fold reduction after *HpaII* digestion for 70 different digestions of the DNA of 28 female MDS patients and 1 male healthy donor. The difference before and after digestion was not significant in four digestions of the DNA of granulocytes of two patients. In the other 66 cases a significant reduction (more than fivefold) of XE169 DNA was observed after *HpaII* digestion.

of malignant cells. Two samples were biopsies taken from lymph nodes of two patients with mantle cell lymphoma. These samples contained more than 95% of malignant cells. Two other samples were obtained from patients with colon carcinoma. In both samples the amount of malignant cells was 60%. Although the amount of DNA extracted from these samples was less than the amount typically obtained from blood samples, both the XE169 and HUMARA PCRs were successful before and after *HpaII* digestion. In both colon carcinoma samples the reduction of XE169 DNA after *HpaII* digestion was less than 10-fold. This led, in combination with the low percentage of tumor cells in these samples, to a polyclonal XCIP ratio instead of the expected skewed XCIP ratio. For one of the lymphoma samples the reduction of XE169 DNA was 49-fold after *HpaII* digestion, corresponding with a monoclonal XCIP pattern after HUMARA PCR. The other lymphoma sample showed a 4.4-fold reduction of XE169 DNA after *HpaII* digestion. In this case the XCIP ratio after HUMARA PCR was skewed, but not fully monoclonal (Table 1). These results show the necessity of monitoring the *HpaII* digestion, especially in paraffin-embedded tissue.

Discussion

We have developed a new, quantitative way to monitor the efficiency of digestion of DNA with the *HpaII* restriction enzyme in the context of clonality analysis with the HUMARA PCR. This method is based on measuring the

Table 1. Reduction of XE169 DNA and XCIP Ratios after *HpaII* Digestion in DNA from Paraffin-Embedded Tissue

Tissue	% Tumor cells	XE169 reduction	XCIP ratio
Non-Hodgkin's lymphoma no. 1	>95	49-fold	9:91
Non-Hodgkin's lymphoma no. 2	>95	4.4-fold	76:24
Colon carcinoma no. 1	60	4.0-fold	56:44
Colon carcinoma no. 2	60	7.1-fold	59:41

reduction after digestion of a gene that escapes X-inactivation (XE169). We found a differential reduction in the amount of XE169 copies in granulocytes and monocytes in two patients after *HpaII* digestion. In both patients the lack of XE169 reduction in granulocytes correlated with a polyclonal XCIP measured with HUMARA PCR. In contrast, the monocytes, originating from the same myeloid precursor cells as granulocytes, showed a clonal XCIP and *HpaII* digestibility in both patients. This indicated that the lack of *HpaII* digestion in the DNA from the granulocytes was probably because of poor DNA quality and that the XCIP was falsely polyclonal in these cells.

In the blood samples of all 30 individuals tested, the monitoring of *HpaII* digestion efficiency was feasible by comparing the amount of XE169 DNA before and after digestion. A significant reduction in XE169 DNA was observed in 66 *HpaII* digestions of DNA from 28 different female MDS samples and two healthy donors. We found a large variation in the reduction of XE169 copies after *HpaII* digestion in individual samples. This was not dependent on the performance of the *HpaII* digestion, as independent digestions of the same sample showed much less variation. The large variation in individual samples may be caused by a difference of DNA quality between samples.

The *HpaII* digestion efficiency was also monitored for DNA from four archival, paraffin-embedded tissue samples. In one sample adequate *HpaII* digestion monitored by XE169 DNA reduction corresponded with the expected monoclonal XCIP ratio. The other three samples showed a XE169 DNA reduction of less than 10-fold after *HpaII* digestion, indicating an inefficient reaction. In another lymphoma sample, the corresponding HUMARA PCR showed a skewed XCIP ratio instead of the monoclonal pattern expected from the more than 95% of tumor cells in this sample. In the two colon carcinoma samples the inefficient *HpaII* digestion combined with the low tumor cell percentage (60%) led to polyclonal XCIP ratios after HUMARA PCR. These results imply that DNA from paraffin-embedded tissues may be of poorer quality than DNA from blood samples and stress the importance of monitoring of *HpaII* digestion efficiency, especially in paraffin-embedded material.

Incomplete *HpaII* digestion leads to false polyclonal background signal in the XCIP determined by HUMARA PCR. The magnitude of the resulting error is dependent on the actual XCIP. If the reduction in DNA is 10-fold then an XCIP ratio of 100:0 would become 91:9, a ratio of

75:25 would become 70:30 and a ratio of 50:50 would remain the same. As the margin of error for the XCIP determined by HUMARA PCR is ~10%,²¹ a 10-fold reduction of DNA after *HpaII* digestion would suffice in this setting. Reductions of less than 10-fold diminish the accuracy of the XCIP determination as has been shown in two blood and three paraffin-embedded tissue samples.

Clonality analysis based on XCIPs is generally a reliable technique. For most tissues in the human body nonrandom, or skewed XCIPs that are not related to clonality are found in only a minority of individuals. This can be identified quite easily by analysis of normal cells adjacent to the suspected sample.^{2,9,22-25} An exception is the mammary gland, which is composed of monoclonal patches in healthy donors forestalling the distinction between hyperplastic and neoplastic lesions based on clonality.²⁶ Excessively skewed XCIPs are also found in hematopoietic cells of healthy elderly females (~40% of individuals older than 60 years of age).²⁷⁻²⁹ This acquired skewing is mainly because of selective pressure for one or more genetic differences between the two X-chromosomes.³⁰⁻³²

Apart from false clonality, false polyclonality may be observed. This can be because of the presence of contaminating normal cells such as infiltrating inflammatory cells. Recently it has been shown that unstable methylation may also cause false polyclonality in aberrant crypt foci of the human colon.³³ A third, technical reason for false polyclonality is incomplete *HpaII* digestion of the active, unmethylated X-chromosomes. We have shown that this can be identified by measuring the reduction after digestion of a gene that escapes X-chromosome inactivation.

We conclude that monitoring the efficiency of the *HpaII* digestion in a HUMARA PCR setting is both necessary and feasible. With quantitative real-time PCR on the XE169 gene false polyclonality because of ineffective *HpaII* digestion can be eliminated.

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References

1. Lyon MF: Gene action in the X-chromosome of the mouse (*mus musculus* L.). *Nature* 1961, 190:372-373
2. Jimbo H, Hitomi Y, Yoshikawa H, Yano T, Momoeda M, Sakamoto A, Tsutsumi O, Taketani Y, Esumi H: Evidence for monoclonal expansion of epithelial cells in ovarian endometrial cysts. *Am J Pathol* 1997, 150:1173-1178
3. Willman CL, Busque L, Griffith BB, Favara BE, McClain KL, Duncan MH, Gilliland DG: Langerhans' cell histiocytosis (histiocytosis X)—a clonal proliferative disease. *N Engl J Med* 1994, 331:154-160
4. Niho S, Yokose T, Suzuki K, Kodama T, Nishiwaki Y, Mukai K: Monoclonality of atypical adenomatous hyperplasia of the lung. *Am J Pathol* 1999, 154:249-254
5. Wada H, Enomoto T, Fujita M, Yoshino K, Nakashima R, Kurachi H, Haba T, Wakasa K, Shroyer KR, Tsujimoto M, Hongyo T, Nomura T, Murata Y: Molecular evidence that most but not all carcinosarcomas

- of the uterus are combination tumors. *Cancer Res* 1997, 57:5379–5385
6. Niho S, Suzuki K, Yokose T, Kodama T, Nishiaki Y, Esumi H: Monoclonality of both pale cells and cuboidal cells of sclerosing hemangioma of the lung. *Am J Pathol* 1998, 152:1065–1069
7. Zhu JJ, Leon SP, Folkert RD, Guo SZ, Wu JK, Black PM: Evidence for clonal origin of neoplastic neuronal and glial cells in gangliogliomas. *Am J Pathol* 1997, 151:565–571
8. Rabkin CS, Janz S, Lash A, Coleman AE, Musaba E, Liotta L, Biggar RJ, Zhuang Z: Monoclonal origin of multicentric Kaposi's sarcoma lesions. *N Engl J Med* 1997, 336:988–993
9. Lee SD, Shroyer KR, Markham NE, Cool CD, Voelkel NF, Tudor RM: Monoclonal endothelial cell proliferation is present in primary but not secondary pulmonary hypertension. *J Clin Invest* 1998, 101:927–934
10. Chang HW, Leong KH, Koh DR, Lee SH: Clonality of isolated eosinophils in the hypereosinophilic syndrome. *Blood* 1999, 93:1651–1657
11. Mach-Pascual S, Legare RD, Lu D, Kroon M, Neuberg D, Tantravahi R, Stone RM, Freedman AS, Nadler LM, Gribben JG, Gilliland DG: Predictive value of clonality assays in patients with non-Hodgkin's lymphoma undergoing autologous bone marrow transplant: a single institution study. *Blood* 1998, 91:4496–4503
12. 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, 51:1229–1239
13. Paradis V, Dargere D, Bonvoust F, Rubbia-Brandt L, Ba N, Bioulac-Sage P, Bedossa P: Clonal analysis of micronodules in virus C-induced liver cirrhosis using laser capture microdissection (LCM) and HUMARA assay. *Lab Invest* 2000, 80:1553–1559
14. Wu J, Ellison J, Salido E, Yen P, Mohandas T, Shapiro LJ: Isolation and characterization of XE169, a novel human gene that escapes X-inactivation. *Hum Mol Genet* 1994, 3:153–160
15. Wu J, Salido EC, Yen PH, Mohandas TK, Heng HH, Tsui LC, Park J, Chapman VM, Shapiro LJ: The murine XE169 gene escapes X-inactivation like its human homologue. *Nat Genet* 1994, 7:491–496
16. Miller AP, Willard HF: Chromosomal basis of X chromosome inactivation: identification of a multigene domain in Xp11.21-p11.22 that escapes X inactivation. *Proc Natl Acad Sci USA* 1998, 95:8709–8714
17. Janssen JW, Buschle M, Layton M, Drexler HG, Lyons J, van den Berghe H, Heimpel H, Kubanek B, Kleihauer E, Mufti GJ: Clonal analysis of myelodysplastic syndromes: evidence of multipotent stem cell origin. *Blood* 1989, 73:248–254
18. Tefferi A, Thibodeau SN, Solberg JR LA: Clonal studies in the myelodysplastic syndrome using X-linked restriction fragment length polymorphisms. *Blood* 1990, 75:1770–1773
19. Karasawa M, Tsukamoto N, Sakai H, Okamoto K, Maehara T, Naruse T, Morita K, Sato S: Clinical outcome in three patients with myelodysplastic syndrome showing polyclonal hematopoiesis. *Acta Haematol* 1999, 101:46–49
20. van Dijk JP, Heuvel L, Stevens-Linders E, Jansen JH, Mensink EJB, Raymakers RAP, de Witte T: Acquired skewing of Lyonization remains stable for a prolonged period in healthy blood donors. *Leukemia* 2002, 16:362–367
21. Gale RE, Mein CA, Linch DC: Quantification of X-chromosome inactivation patterns in haematological samples using the DNA PCR-based HUMARA assay. *Leukemia* 1996, 10:362–367
22. Asplund A, Guo Z, Hu X, Wassberg C, Ponten F: Mosaic pattern of maternal and paternal keratinocyte clones in normal human epidermis revealed by analysis of X-chromosome inactivation. *J Invest Dermatol* 2001, 117:128–131
23. Endo Y, Sugimura H, Kino I: Monoclonality of normal human colonic crypts. *Pathol Int* 1995, 45:602–604
24. Gaffey MJ, Iezzoni JC, Weiss LM: Clonal analysis of focal nodular hyperplasia of the liver. *Am J Pathol* 1996, 148:1089–1096
25. Mutter GL, Boynton KA: X chromosome inactivation in the normal female genital tract: implications for identification of neoplasia. *Cancer Res* 1995, 55:5080–5084
26. Diallo R, Schaefer KL, Poremba C, Shivazi N, Willmann V, Buerger H, Dockhorn-Dworniczak B, Boecker W: Monoclonality in normal epithelium and in hyperplastic and neoplastic lesions of the breast. *J Pathol* 2001, 193:27–32
27. Busque L, Mio R, Mattioli J, Brais E, Blais N, Lalonde Y, Maragh M, Gilliland DG: Nonrandom X-inactivation patterns in normal females: Lyonization ratios vary with age. *Blood* 1996, 88:59–65
28. Champion KM, Gilbert JG, Asimakopoulos FA, Hinshelwood S, Green AR: Clonal haemopoiesis in normal elderly women: implications for the myeloproliferative disorders and myelodysplastic syndromes. *Br J Haematol* 1997, 97:920–926
29. Gale RE, Fielding AK, Harrison CN, Linch DC: Acquired skewing of X-chromosome inactivation patterns in myeloid cells of the elderly suggests stochastic clonal loss with age. *Br J Haematol* 1997, 98:512–519
30. Vickers MA, McLeod E, Spector TD, Wilson IJ: Assessment of mechanism of acquired skewed X inactivation by analysis of twins. *Blood* 2001, 97:1274–1281
31. Abkowitz JL, Taboada M, Shelton GH, Catlin SN, Guttorp P, Kiklevich JV: An X chromosome gene regulates hematopoietic stem cell kinetics. *Proc Natl Acad Sci USA* 1998, 95:3862–3866
32. Christensen K, Kristiansen M, Hagen-Larsen H, Skytthe A, Bathum L, Jeune B, Andersen-Ranberg K, Vaupel JW, Orstavik KH: X-linked genetic factors regulate hematopoietic stem-cell kinetics in females. *Blood* 2000, 95:2449–2451
33. Sakurazawa N, Tanaka N, Onda M, Esumi H: Instability of X chromosome methylation in aberrant crypt foci of the human colon. *Cancer Res* 2000, 60:3165–3169