

Temperature dependent γ -H2AX binding to DNA

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ABSTRACT: γ -H2AX, the serine 139 phosphorylated form of histone H2AX, is one of the earliest DNA double-strand breaks repair responses. Therefore, the quantity of γ -H2AX-bound DNA usually reflects the extent of DNA damage and consequently, mutagenic potential. The purpose of this study was to assess the cellular evidence regarding whether and how temperature is related to mutation. This study applies our novel assay technique to measure the quantity of γ -H2AX-bound DNA by PCR. Chromatin immunoprecipitation technique was applied to select for the γ -H2AX-bound DNA complex. Then, the amount of precipitated DNA was measured by long interspersed element-1 (LINE-1) repetitive sequences real-time PCR. We observed a positive correlation between the amount of precipitated LINE-1 sequences and the exposure to temperature. The quantity of γ -H2AX-bound DNA increased significantly when HeLa was incubated in 47 °C for 30 min. In contrast, incubation at 4 °C for 30 min reduced the γ -H2AX-bound DNA quantity. Precise quantification of γ -H2AX-bound DNA will be a tool to prove whether there is a linkage between temperature and mutagenic effect.

KEYWORDS: γ -H2AX, DNA double-strand breaks, DSBs, ChIP, LINE-1, real-time PCR, carcinogenic potential

INTRODUCTION

DNA double-strand breaks (DSBs) are biologically significant lesions leading to mutations which are a cause of cancer and hereditary disease¹. DSBs are induced by several environmental agents and also occur spontaneously from cellular processes². In mammalian cells, DSBs can be repaired *via* non-homologous end joining or homologous recombination depending on the cell cycle^{3–5}. γ -H2AX, the serine 139 phosphorylated form of histone H2AX, is one of the earliest DSB repair responses^{6,7}. Therefore, a technique for γ -H2AX-bound DNA detection and quantification is a crucial tool to understand the role of DSBs and DSB repair in human diseases, particularly hereditary and cancer developmental process⁸. To detect the phosphorylated form of H2AX as foci corresponding to each DSB site in nuclei, an immunofluorescence staining method with antibodies specific for γ -H2AX has been successfully used^{9–13}. γ -H2AX increases in a variety of conditions in relation to DSBs generation processes, including radiation^{14,15} and high temperature^{16,17}. Although the exact role of heat-directed DSBs has been argued over¹⁸, the fact that heat can induce γ -H2AX foci leads to the possibility of association between temperature and carcinogenesis¹⁹. γ -H2AX not only marks DSB lesions but also may play an important role in DSB repair. Depletion in H2AX or

phosphorylation of H2AX has been demonstrated to cause genome instability and consequently carcinogenesis. For example, H2AX-deficient mice and mouse embryonic stem cells were hypersensitive to ionizing radiation (IR) and showed elevated levels of chromosome instability^{20–24}. Additionally, H2AX knock out mice were prone to develop tumours^{20,22,25}.

In this study, we developed a novel technique to quantify the γ -H2AX bound DNA by PCR and called this new assay “H2AX qPCR.” We used the chromatin immunoprecipitation technique (ChIP)²⁶ to select the γ -H2AX-bound DNA complex and combined it with interspersed repetitive sequence PCR^{27–30}. We used real-time quantitative PCR^{31,32} to detect and to quantify the γ -H2AX-bound DNA that occurs randomly throughout the genome. The interspersed repetitive sequences in this protocol were LINE-1 repetitive sequences³³. This technique improved quantitative accuracy compared to the conventional immunofluorescence staining method. Therefore, we applied the technique to evaluate the γ -H2AX-bound DNA under different temperatures.

MATERIALS AND METHODS

Cell culture

HeLa (cervical cancer) cells were grown in DMEM containing 10% FBS. Before further study, cells were synchronized at G 0 phase by culturing in

a serum-deprived medium for 24 h. The cells were maintained at 37 °C in a conventional humidified CO₂ incubator. For temperature treatments, HeLa cells were incubated at 4 °C or 47 °C for 30 min.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed essentially as previously described with some modifications³⁴. Histone cross-linking to DNA was induced by adding formaldehyde directly to the culture medium at a final concentration of 1%, with incubation at 37 °C for 10 min. After stopping the reaction with glycine (0.125 M final concentration) for 5 min at room temperature, adherent cells were washed twice with ice-cold PBS, and then scraped into ice-cold PBS containing Halt protease inhibitor cocktail (Pierce, Rockford, IL, USA). Nuclei were isolated by resuspending the cell pellet in cell lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, containing Halt protease inhibitor cocktail) and incubated on ice for 20 min. Intact nuclei were collected by centrifugation at 3210 g for 5 min at 4 °C, resuspended in nuclear lysis buffer (1% SDS, 50 mM Tris-HCl pH 8.1, 10 mM EDTA, containing Halt protease inhibitor cocktail), and incubated on ice for 10 min. Chromatin was sheared with an ultrasonic sonicator at 30% power output for four 30 s intervals on ice to an average size of 500–1 000 bp. After centrifugation at 21,720 g for 10 min at 4 °C, the chromatin solution was diluted 10-fold with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM

NaCl, containing Halt™ Protease Inhibitor Cocktail kit) and then precleared for 30 min at 4 °C with protein G Plus-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) with rotation. The agarose beads were pelleted for 1 min at 180 g and the chromatin fragments were immunoprecipitated overnight at 4 °C with Anti-phospho-histone H2AX monoclonal antibody (Upstate, Charlottesville, VA, USA) or normal mouse IgG antibody as a negative control (Santa Cruz Biotechnology) on a rotator. Protein-DNA-antibody complexes were isolated by the addition of protein G Plus-Agarose. After 2 h, agarose beads were collected by centrifugation at 120 g for 1 min, washed once each in 500 mM, 550 mM and 600 mM high-salt wash buffers (0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl pH 8.0, 2 mM EDTA, 500–600 mM NaCl), and twice in wash buffer (100 mM Tris-HCl pH 8.0, 500 mM 1% Nonidet P-40, 1% deoxycholic acid). Complexes were eluted with elution buffer (50 mM NaHCO₃, 1% SDS) for 15 min at room temperature. Cross-links were reversed by adding NaCl (200 mM final concentration) and RNA was removed by adding 10 mg/ml of RNase A, followed by incubation for 4 h at 65 °C, and then precipitated overnight with ethanol. Samples were deproteinized with proteinase K. After phenol/chloroform extraction, the DNA was precipitated with ethanol. The precipitated sample was subjected to real-time 5'LINE-1 PCR to measure the quantity of LINE-1 sequence

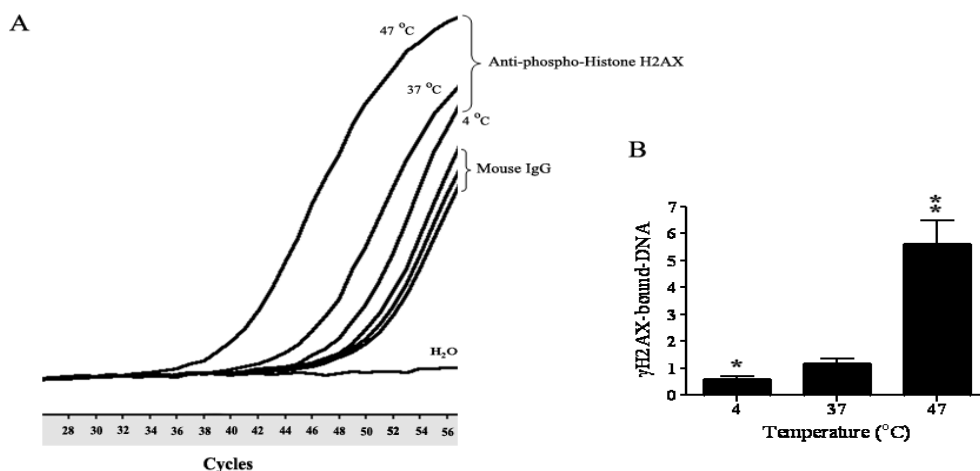


Fig. 1 The quantity of γ -H2AX-bound DNA. (A) An example of real-time LINE-1s PCR from ChIP of anti-phospho-histone H2AX monoclonal antibody or normal mouse IgG antibody bound DNA and water. (B) γ -H2AX-bound DNA from HeLa cells in 4 °C, 37 °C, and 47 °C media. The quantity of γ -H2AX-bound-LINE-1 sequences was used to calculate the relative amount of γ -H2AX-bound genomic DNA. The units are Mb per cell. * P <0.05, ** P <0.001 (paired 2-tailed t-test).

Real-time PCR

Quantification of the amount of immuno-precipitated 5'LINE-1 sequences was carried out by real-time PCR using a QuantiTect SYBR Green PCR Kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. Briefly, 1× QuantiTect SYBR Green PCR Master Mix, 0.2 μM forward primer (L1.2HpaIIRFLPF:5'-CTCCCAGCGTGAGC GAC-3'), and 0.2 μM reverse primer (5'LIDSIP1st-AC TCCCTAGTGAGATGAACCC G-3') were used for each PCR assay. The PCR program was initiated at 95 °C for 15 min to activate the HotStarTaq DNA polymerase, followed by 50 thermal cycles of 15 s at 95 °C, 20 s at 57 °C, and 20 s at 72 °C. A melting curve test (68 °C) was always carried out after the final reaction step to confirm that appropriate amplification products were obtained. Each sample was analysed in triplicate PCR reactions. All reactions were run on a LightcyclerTM instrument (Roche Applied Science). Control HeLa genomic DNA was used as a positive control and for calculating the precipitated DNA quantity from real-time PCR.

Quantification of the γ-H2AX-bound DNA

From real-time PCR, the amount of γ-H2AX-bound DNA was calculated using:

$$\text{Amount of } \gamma\text{-H2AX-bound DNA per cell (Mb per cell)} = \frac{N(A-B)}{C},$$

where N is the amount of mega base pairs (Mb) in one cell (which corresponds to the whole genome), A is the quantity of γ-H2AX-bound DNA, B is the quantity of mouse IgG antibody-bound DNA, and C is the total starting DNA input per one reaction of real-time PCR. The γ-H2AX-bound DNA, measured mouse IgG antibody-bound DNA, and total starting DNA input per one reaction are measured in the same units. For practical purposes, this unit is a genome. The units of γ-H2AX-bound DNA is Mb per cell.

RESULTS

We investigated the association between temperatures and γ-H2AX-bound DNA by using our novel method called H2AX qPCR. In this assay, γ-H2AX-bound DNA was obtained by ChIP using a γ-H2AX antibody. Subsequently, the bound LINE-1 sequences were detected and quantified by real-time PCR with both primers located at the 5' region of LINE-1. The result of H2AX qPCR is shown in Fig. 1A and indicates that the amount of γ-H2AX-bound DNA was measured by PCR. Both

γ-H2AX and interspersed repetitive sequences are randomly distributed in our genome^{33,35,36}. Therefore, the amount of γ-H2AX-bound LINE-1 should be represented in direct proportion to the γ-H2AX-bound DNA. Nevertheless, there are thousands more copies of LINE-1s than unique DNA sequences. Consequently, nonspecific binding of repeat sequences cannot be completely excluded from the experiments. To estimate the extent of this nonspecific binding, we included a nonspecific antibody, normal mouse IgG antibody, as a mock experiment to compare with the test anti-γ-H2AX monoclonal antibody. Our results indicate that the mock experiments usually yielded a significantly lower quantity of precipitated DNA than the test (Fig. 1A). We did not apply unphosphorylated H2AX antibody as a negative control. First, the anti-γ-H2AX monoclonal antibody was specific to the phosphorylated form of H2AX⁹⁻¹³. Second, the aim of this technique was to measure γ-H2AX-bound DNA that represents the quantity of DSBs in a genome or a cell. Since there is no information regarding the total amount of unphosphorylated H2AX per cell, the measurement of γ-H2AX-bound DNA in relation to the unphosphorylated H2AX bound DNA may provide biased information regarding the extent of DSBs. Finally, H2AX is a subclass of eukaryotic histone proteins, densely distributed throughout the genome. Immuno-precipitating the proteins may yield a significant quantity of the genome, a million times larger than γ-H2AX-bound DNA. This would lead to a very wide margin of error.

Previous studies^{16,17} demonstrated increased γ-H2AX foci in cells under hyperthermic conditions (45–47 °C) and suggested that heat may induce DSBs. However, the possibility that heat alters the cellular repair response to endogenous DSBs is another reasonable hypothesis. Here we applied our new established technique to evaluate whether we could measure the quantity of temperature-induced γ-H2AX-bound DNA alteration. Our qPCR technique yields precise quantities. Because there is a significant amount of γ-H2AX-bound DNA under physiological conditions (37 °C), we investigated how a lower temperature would affect the quantity of γ-H2AX-bound DNA. We incubated Hela cells at 4 °C, 37 °C and 47 °C for 30 min and evaluated the quantity of γ-H2AX-bound DNA by using H2AX qPCR. We observed a significant increase in H2AX-bound DNA when the temperature increased (Fig. 1B). Therefore, this technique successfully identified the increased γ-H2AX-bound DNA from hyperthermia. In contrast, a smaller amount of γ-H2AX-bound DNA from hypothermia was observed (Fig. 1B).

DISCUSSION

DSBs are important DNA lesions. Failure to repair the lesions will lead to apoptosis, and errors in DNA repairs can lead to mutations. The most crucial biological lesions lead to hereditary diseases and cancer. Therefore, it is important to evaluate the causes of DSBs and the conditions that determine cellular effectiveness in DSB repair. Alteration of γ -H2AX-bound DNA at higher and lower temperatures is an important finding. Even though our physiological condition usually maintains cells at 37 °C, many cells such as skin and the upper gastrointestinal tract are often exposed to lower and higher temperatures. Consequently, our finding could be important in explaining the natural consequences of abnormal temperature to our cells. This study confirmed that heat can increase γ -H2AX-bound DNA. Moreover, we are the first to demonstrate that reduced temperature can decrease γ -H2AX-bound DNA. There are several possible mechanisms by which lower temperatures can reduce endogenous H2AX bound DNA. The lower temperature may reduce either heat-induced DSBs or the enzymatic activity of DSB repair proteins. Additionally, temperature may alter the chromatin configuration and consequently change the DNA repair response. Further investigation to prove these mechanisms is crucial since each possibility leads to a different mutation potential. If lower temperature reduces endogenous DSBs, lower temperature may play a role in mutation prevention. On the contrary, if lower temperature reduces DNA repair activity, low temperature may contribute to genomic instability and consequently alter the mutation rate.

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