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Heat induces gene amplification in cancer cells

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ABSTRACT

Background: Hyperthermia plays an important role in cancer therapy. However, as with radiation, it can cause DNA damage and therefore genetic instability. We studied whether hyperthermia can induce gene amplification in cancer cells and explored potential underlying molecular mechanisms.
Materials and methods: (1) Hyperthermia: HCT116 colon cancer cells received water-submerged heating treatment at 42 or 44 °C for 30 min; (2) gene amplification assay using *N*-(phosphoacetyl)-L-aspartate (PALA) selection of cabamyl-P-synthetase, aspartate transcarbarmylase, dihydro-orotase (*cad*) gene amplified cells; (3) southern blotting for confirmation of increased *cad* gene copies in PALA-resistant cells; (4) γH2AX immunostaining to detect γH2AX foci as an indication for DNA double strand breaks.
Results: (1) Heat exposure at 42 or 44 °C for 30 min induces gene amplification. The frequency of *cad* gene amplification increased by 2.8 and 6.5 folds respectively; (2) heat exposure at both 42 and 44 °C for 30 min induces DNA double strand breaks in HCT116 cells as shown by γH2AX immunostaining.
Conclusion: This study shows that heat exposure can induce gene amplification in cancer cells, likely through the generation of DNA double strand breaks, which are believed to be required for the initiation of gene amplification. This process may be promoted by heat when cellular proteins that are responsible for checkpoints, DNA replication, DNA repair and telomere functions are denatured. To our knowledge, this is the first study to provide direct evidence of hyperthermia induced gene amplification.

1. Introduction

Hyperthermia, the elevation of tumor temperature to a supra-physiologic level in the range of 40–44 °C, is a well-established treatment that uses physical energy to precipitate damage in tumor tissues. Hyperthermia combined with chemotherapy and radiotherapy has improved clinical outcome of cancers. It not only has direct killing effect on the tumor cells, but also can improve the body's immune response and inhibit tumor metastasis. It has become an effective cancer therapy in conjunction with surgery, radiotherapy and chemotherapy.

Cancer is a genetic disease resulting from accumulation of mutations in genes that control cell death and proliferation [1]. Although hyperthermia plays an important role in cancer therapy, it can cause DNA damage and therefore potentially promotes carcinogenesis and tumor progression especially when it occurs in the presence of known carcinogens, such as radiation or chemical carcinogens [2]. DNA damage can cause genetic instability which is a hallmark of cancer and contributes to tumor progression and development of resistance to chemo or radiation therapy [1]. Gene amplification is a form of genetic instability. By definition, gene amplification is an increase in the number of copies of a restricted region of a chromosome [3]. Gene amplification is prevalent in some tumors and is associated with over expression of gene(s), which is a common mechanism underlying oncogenic activation.

Clinically, gene amplification has prognostic and diagnostic usefulness, and is a mechanism of acquired drug resistance [3]. Amplification of oncogenes such as *cyclin, her2/neu, akt2*, and *myc* have been reported in a variety of human cancers. A well known mechanism underlying resistance to methotrexate is through amplification of *dhfr* gene in cancer cells. To date, there are no reports showing that hyperthermia leads to gene amplification. Our study provides direct evidence showing that prolonged thermal exposure to 42 and 44 °C induced gene amplification in HCT116 cancer cells.

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The most likely underlying mechanism is the generation of DNA double strand breaks (DSB) by thermal exposure.

2. Materials and methods

2.1. Cell culture

HCT116 colon cancer cells were obtained from ATCC (Manassas, VA) and were cultured in DMEM medium (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum(PAA, Linz, Austria) and 1% penicillin/streptomycin (100 U/mL and 100 g/mL; PAA, Linz, Austria) at 37 °C with 5% CO_2 and humidity-saturated incubator.

2.2. Hyperthermia treatment

Cells received water-submerged heating treatment at 42 or 44 °C for 30 min. Briefly, the culture plate was sealed in a plastic bag and then immersed in a digital water bath (Shel Lab, Cornelius, OR). The control group cells were maintained at 37 °C.

2.3. Determination of 50% lethal dosage (LD_{50}) of PALA in HCT116 cells

Six thousand cells/well were seeded into a 24-well plate and grown in the presence of a titrated ascending concentration of *N*-(phosphoacetyl)-L-aspartate (PALA). Once the cells in the well free of PALA grew to confluence, the cells were washed once with PBS, and then stained with methylene blue solution (0.4% methylene blue dissolved in 30% methanol solution) for 20 min. Methylene blue was extracted with 1 ml 1% acetic acid solution after being washed with distilled water 3–4 times. The absorbance OD650 was measured at λ = 650 nm and then a graph was made to determine LD_{50}, the concentration that inhibited 50% cell growth.

2.4. Gene amplification assays

Clonogenic assay were used to measure the frequency of gene amplification in HCT116 cells. The selective agent for *cad* gene amplification, PALA, was obtained from the Drug Synthesis Branch, Division of Cancer treatment, National Cancer Institute. To estimate the frequency of gene amplification, we determined the LD_{50} (the dose that inhibited 50% of treated cell growth) for PALA in the HCT116 cell line. For estimation of resistant clones, about $1–2 \times 10^6$ cells were inoculated into a 10 cm Petri dish. Cells were then selected by introduction of $3.5 \times LD_{50}$ concentration of PALA. Resistant colonies typically appeared within 2–3 weeks. These colonies were counted after fixation and staining with Crystal violet (CV) staining fixative (0.5% CV in 80% methanol). Frequency of amplification is expressed as the number of resistant colonies relative to the number of colonies formed without PALA.

2.5. Southern blotting

Genomic DNA from cultured cells was isolated by the phenol–chloroform extraction. Ten microgram DNA was digested with *Pst*I endonuclease overnight. Digested DNA was subjected to slow electrophoresis through 0.8% agarose gel. The agarose gel was then rinsed with water and treated with denaturation buffer (1.5 M NaCl, 0.5 M NaOH) for 1 h, followed with neutralization buffer (1.5 M NaCl, 0.5 M tris, pH 7.5) for 1 h. DNA was subsequently transferred to a nylon membrane using 10× SSC buffer (1.5 M NaCl, 150 mM Na Citrate, pH 7.0). A purified 1.5 kb PCR fragment corresponding to CAD sequence (2645–4146) was prepared and used as template for the generation of ^{32}P-dCTP labeled probes. Probes were generated with Ready-To-Go DNA labeling Beads (-dCTP) (Amersham) following manufacture instruction. Unincorporated nucleotides and short fragments (<20 bases) were then removed using a ProbeQuant G-50 Micro column. DNA prehybridization and hybridization were performed with a same hybridization buffer (88 g/L NaCl, 44 g/L Na Citrate, 50% formamide, 10 mg/ml ssDNA, 250 mM Tris, pH 7.5, 500 mM EDTA, 0.5% Ma pyrophosphate, 5% SDS, 1% polyvinylpyrolidone-400,000, 1% Ficoll, 1% BSA). DNA probes were added to hybridization solution to a final 1.5 million cpm/mL ^{32}P radioactivity, and incubated with membrane with a continuous motion in a hybridization oven (42 °C) for overnight. Membrane was rinsed and then washed with prewarmed wash buffer (88 g/L NaCl, 44 g/L Na Citrate, 0.1% SDS) for 15 min twice at 55 °C. Membrane was wrapped with SARAN membrane and expose three days at −80 °C.

2.6. γH2AX immunostaining

2×10^5 Cells were seeded in a Lab-Tek chamber (Cole-Parmer,Vernon Hills, IL). After immersion in 44 °C water bath for 30 min, cells were rinsed in PBS, then fixed in 4% paraformaidehyde for 20 min, rinsed again in PBS and then permeabilized in 0.2% Triton-X 100. After blocking with blocking bovine serum albumin (BSA) for 1 h, the cells were incubated for overnight at 4 °C with mouse anti-human γH2AX antibody (1:500) (Bethyl Laboratories Inc, Montgome, TX, USA, 0.1 μg/ml). The cells were rinsed in PBS and incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit Immunoglobulin (1:500, Abcam, Cambridge, MA). 4,6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO) was added to the cells to stain the nuclei and incubated for another 15 min. The cells were examined under an Olympus IX71 fluorescent microscope (Olympus, Tokyo, Japan). The cells with foci number >10/cell were defined as positive [4].

3. Results

3.1. Hyperthermia induced gene amplification in HCT116 cells

Gene amplification is a commonly used measure of genomic instability in transformed cells [5–7]. It has been shown previously that ionizing radiation can increase the frequency of gene amplification significantly [8]. The frequencies of gene amplification were estimated at one gene locus, the cabamyl-P-synthetase, aspartate transcarbarmylase, dihydro-orotase (*cad*) gene. Amplifications of the *cad* genes will allow the cells to be resistant to PALA. Therefore, it is possible to estimate the frequency of the cells that have undergone *cad* gene amplifications by quantifying the number of cell colonies that are resistant to PALA. We first determined the 50% lethal dosage of PALA in HCT116 cells. Cells were grown in a titrated concentration of PALA. As shown in Fig. 1A, the calculated concentration that inhibit cell growth to 50% is 33.7 μM. Gene amplification assay were performed using PALA selection at a concentration of $3.5 \times LD_{50}$ (120 μM) according to previous reports [9]. As shown in Fig. 1B, hyperthermia and radiation significantly induced elevation of frequency of PALA-resistant clones which was about 2.8, 6.5 and 13.3-fold higher in 42, 44 °C-heated and irradiated cells respectively than in control cells. Amplification of the *cad* gene in PALA-resistant clones was confirmed by Southern Blotting. In most of the clones examined, *cad* gene dosage was increased 2–3 folds in these cells (Fig. 1C).

3.2. Hyperthermia induced DNA DSB in HCT116 cells

The exact mechanism of gene amplification is not completely understood. However it is clear that DNA double strand break

(DSB) is required for initiation of gene amplification. H2AX is a histone variant which is present and ubiquitously distributed throughout the genome. H2AX is rapidly phosphorylated at serine 139 (γH2AX) upon DSB generation and forms foci at the DSB sites [10,11]. γH2AX is required for recruiting repair and checkpoint proteins to the damaged sites. Because of the close correlation between γH2AX and DSBs, an immunocytochemical assay using antibodies capable of recognizing γH2AX has been developed as a very sensitive and specific indicator for the existence of DNA double strand breaks [12,13].

As shown in Fig. 2, exposure of HCT116 cells to 42 °C for 30 min significantly induced γH2AX foci formation in the nuclei. Heat exposure induced double strand breaks in 85% of the cells. It indicates that hyperthermia may cause gene amplification through inducing DNA double strand breaks which is required to generate gene amplifications.

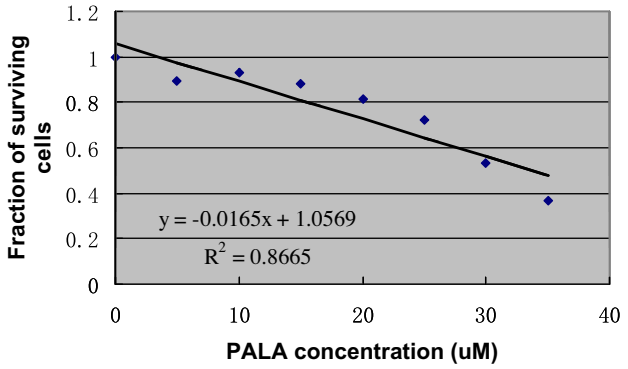


Fig. 1A. PALA LD50 of HCT116 cells. Cells were grown in the presence of titrated ascending concentrations of PALA, 0, 5, 10, 15, 20, 25, 30, 35 μM. The growth/survival fraction were determined by methylene blue staining. LD50 was calculated from the equation derived from the growth/survival curve, which is 33.7 μM.

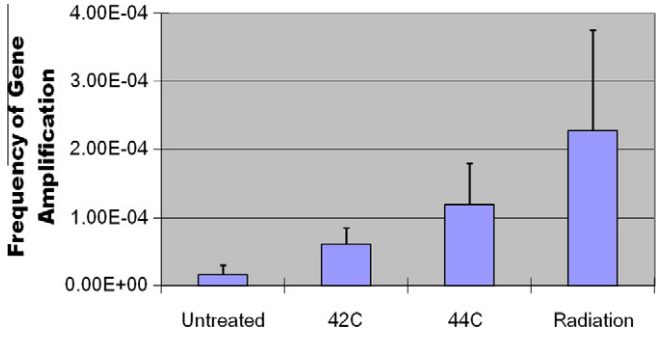


Fig. 1B. HCT116 cells were untreated, treated at 42 or 44 °C for 30 min or treated with 3 Gy X-ray irradiation. Gene amplification assay using PALA was conducted after the cells recovered from treatments.

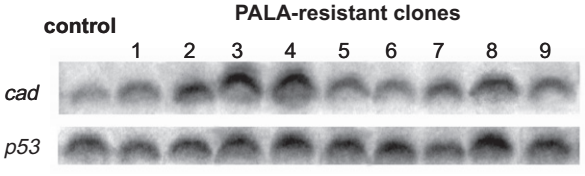
4. Discussion

Heat causes both protein denaturation and damage to DNA, however it is not clear which one is the predominant molecular mechanism of cellular damage. It has been reported that heat inhibits DNA replication, transcription and repair [14–17] and induces structural alterations and strand breaks in chromatin DNA [18–21]. There are mixed reports about whether heat shock can cause DSB. Recent studies showed that thermal exposure led to DSB while older studies failed to detect DSB in cells treated with hyperthermia. This discrepancy can be explained by the much improved sensitivity of recent assays that detect DSB, specifically the γH2AX staining for DSB. γH2AX assay is much more sensitive than older assays that use alkaline and neutral sucrose gradients and the pulsed gel electrophoresis method [21–23].

In concert with some recent studies using γH2AX antibody for DSB detection [24,4], we demonstrate that hyperthermia induces DNA double strand breaks. The molecular mechanism of gene amplification is not clearly understood. It is likely to be initiated by a DNA DSB in cells lacking robust checkpoints [25–29]. Those that promote gene amplification includes errors in DNA replication [30], telomere dysfunction [31,32] and fragile sites (defined as chromosomal regions that are late replicating and prone to breakage under conditions of replication stress) [26,27,33].

What are the possible mechanisms underlying heat-induced gene amplification? Hyperthermia can contribute to gene amplification in several ways. First, we have shown that heat exposure leads to DNA DSBs. DSBs can be caused by direct damage to DNA through heat energy activation or indirectly by heat through denaturing of proteins that are responsible for replication and DNA repair. DSBs are frequent in replicating cells and can be generated by collapse of replication forks unable to progress as a result of encountering lesions in the DNA. These DSBs are not immediately repaired due to the dysfunction of DNA repair proteins and therefore provide an opportunity to initiate the amplification process. Second, cellular protein denaturation by heat exposure can inactivate proteins involved in cell cycle checkpoints, DNA replication, DNA repair and telomere function, causing lack of checkpoints, errors in replication and telomere dysfunction, all of which can promote gene amplification.

It is interesting to note that hyperthermia, like radiation, is a double edged sword. Like radiation, hyperthermia plays an important role in cancer treatment. Yet both can cause DNA damage and induce genetic instability which is crucial for tumor progression and development of tumor resistance to cancer therapies.



Clones	Control	1	2	3	4	5	6	7	8	9
Relative *cad* copy #	1.0	1.96	2.02	3.14	2.86	1.89	1.76	2.41	1.98	1.99

Fig. 1C. Southern blot analysis of the relative gene dosage for PALA-resistant HCT116-clones. Photograph of Southern blot results for selected PALA resistant clones (*upper*). Densitometric analysis of the relative copy number of *cad* genes to that of control gene, *p53*, in the PALA-resistant clones by software (Molecular Dynamics) (*lower*).

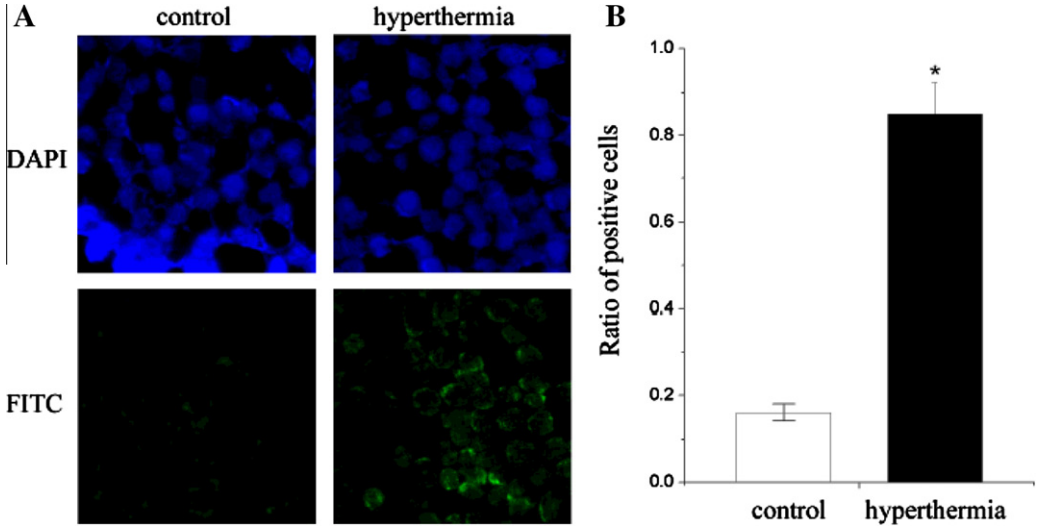


Fig. 2. Each slide chamber seeded with 2 × 105 cells were immersed in 37 °C (control) or 42 °C (hyperthermia) water bath for 30 min. Cells were then rinsed and fixed for immunoflorescent staining using antihuman γH2AX antibody. Cells were examined under an Olympus IX71 fluorescent microscope. Those with foci number >10/cell were counted as γH2AX-positive. (A) Representative immunofluorescent images of γH2AX foci formation in HCT116 cells. (B) Quantitative analysis of the ratio of γH2AX-positive cells. $^{*}p < 0.01$.

To our knowledge, this study is the first to provide direct evidence that heat induces gene amplification in cancer cells. The underlying mechanism is likely to involve generation of DNA DSBs and denaturation of cellular proteins that are responsible for checkpoints, DNA replication, DNA repair and telomere functions.

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