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Research article

In vitro mutation artifacts after formalin fixation and error prone translesion synthesis during PCR

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Abstract

Background: Clinical specimens are routinely fixed in 10% buffered formalin and paraffin embedded. Although DNA is commonly extracted from fixed tissues and amplified by PCR, the effects of formalin fixation are relatively unknown. Formalin fixation is known to impair PCR, presumably through damage that blocks polymerase elongation, but an insidious possibility is error prone translesion synthesis across sites of damage, producing *in vitro* artifactual mutations during PCR.

Methods: To better understand the consequences of fixation, DNA specimens extracted from fresh or fixed tissues were amplified with *Taq* DNA polymerase, and their PCR products were cloned and sequenced.

Results: Significantly more (3- to 4-fold) mutations were observed with fixed DNA specimens. The majority of mutations were transitions, predominantly at A:T base pairs, randomly distributed along the template.

Conclusions: Formalin fixation appears to cause random base damage, which can be bridged during PCR by *Taq* DNA polymerase through error prone translesion synthesis. Fixed DNA is a damaged but "readable" template.

Background

Human tissues are routinely fixed in 10% buffered formalin and paraffin embedded. DNA can be extracted from fixed tissues and amplified by PCR [1,2]. Fixed tissues are commonly analyzed by PCR for both research and clinical applications because they are readily available and their DNA is stable for decades [3]. Unfortunately, PCR is more difficult with DNA extracted from fixed tissues. Prolonged fixation intervals are associated with decreased PCR yields and a progressive inability to amplify longer templates [2].

The effects of formalin fixation on DNA present in tissues are uncertain. Formalin forms monomethylol adducts with nucleotide rings, which are reversible in aqueous solutions typically used to extract DNA [4]. However, less reversible reactions also occur [4]. Base damage may impair PCR by halting polymerase elongation, but an insidious possibility is error prone translesion synthesis [5,6] across sites of damage, producing *in vitro*, artifactual mutations. Errors secondary to translesion bypass are mechanistically distinct from polymerase replication fidelity, reflecting incorporation of the wrong base

opposite a damaged base, versus misincorporation of the wrong base opposite a normal base.

Base damage typically halts DNA replication because most polymerases stall at damaged bases. However, recent studies have discovered that some polymerases allow translesional but error prone replication across sites of DNA damage. Y-family bypass DNA polymerases insert non-templated bases across from damaged bases (translesional sythesis), exhibit low replication fidelity, and lack 3' exonuclease activity [5,6]. *Taq* DNA polymerase, commonly used for PCR, is from the A-family of DNA polymerases, but also lacks 3' exonuclease proofreading activity [7,8] and exhibits translesion synthesis with modified DNA templates [9-11].

The analysis of fixed human tissues may be confounded if formalin damages DNA and *Taq* polymerase performs error prone translesion synthesis across some of this damage. To investigate this possibility, sequences of cloned PCR products were compared between DNA extracted from fresh and fixed tissues.

Methods

Normal colon from a 38 year-old was fixed in 10% buffered formalin for one to seven days and then paraffin embedded. The colectomy was performed for adenocarcinoma and normal colon was obtained near the noninvolved surgical margin. Single 5 micron thin slices of normal appearing mucosa, submucosa, and muscularis were deparaffinized with Clear Right 3 (Richard-Allan Scientific, Kalamazoo, MI) for 3 minutes, followed by three washes in 95% ethanol (one minute each). The deparaffinized tissue was scrapped off the slide and the DNA was extracted for four hours at 56°C (100 mM Tris-HCl, 2 mM EDTA, pH 8.0 with 0.02 mg per ml of Proteinase K), followed by boiling for 5 minutes. For comparison, high molecular weight DNA was extracted from the same fresh tissue using a silica-gel column based method (DNeasy Tissue Kit, Qiagen, Valencia, CA). Therefore, differences in numbers and types of sequence alterations between fixed and fresh tissue should reflect *in vitro* changes because the same tissue is analyzed. Two other routinely processed (i.e. one day of formalin fixation) normal colon clinical specimens were also analyzed. These clinical specimens gave results equivalent to the one day fixed colon and the mutation data were combined.

Approximately 100 ng of DNA was used for PCR (30 to 40 cycles) with primers directed to the mutation cluster region of the APC gene (codons 1145 to 1468). PCR was performed with Ampli*Taq* or Ampli*Taq* Gold DNA polymerase (Applied Biosystems, Foster City, CA) with buffers supplied by the manufacturer, at a 2.5 mM $MgCl_2$ concentration. PCR products were cloned (TOPO TA Cloning kit, Invitrogen, Carlsbad, CA) and individual clones were sequenced with an ABI 377XL automated sequencer.

Mutations were identified by comparison to an APC GenBank reference sequence (GenBank Accession NM_000038.2, gi:21626462). All mutations gave clear sequence traces, or sequencing was repeated for verification. Statistical significance was determined with chi-square or Fisher's exact tests.

Results

The mutation cluster region of the APC gene was amplified with primers producing products from 420 to 1007 base pairs in length. DNA isolated from formalin fixed tissues produced less PCR product relative to DNA isolated from fresh tissue, and longer formalin fixation intervals were associated with decreased PCR product yields and a progressive inability to amplify longer templates (Figure 1). No PCR products were observed with a fixation interval of seven days. Greater PCR yields were observed with Ampli*Taq* Gold, a chemically modified version of Ampli*Taq* DNA polymerase that requires heat for activation, compared to Ampli*Taq* DNA polymerase (data not shown).

Sequences of cloned PCR products revealed mutation frequencies approximately 3- to 4-fold greater ($p < 0.05$) with formalin fixed tissues (Figure 2). There were five mutations out of 20,900 sequenced bases from fresh DNA (0.00024 mutations per base) and 76 mutations out of 80,094 sequenced bases from fixed DNA (0.00095 mutations per base). Mutation frequencies did not significantly vary with template size or length of formalin fixation. There were no significant differences in mutation frequencies between Ampli*Taq* and Ampli*Taq* Gold DNA polymerases (data not shown).

Mutation types were different after fixation, with a predominance (92%) of transition mutations (Figure 3). Small deletions and an addition at simple short repeat sequences were also observed (6.6% of mutations) with fixed DNA (Figure 3). Mutations in the fixed DNA were scattered throughout the template (Figure 3) with no obvious hot spots. Point mutations at A:T base pairs were significantly ($p= 0.034$) more frequent than at G:C pairs in the fixed DNA (2.9 to 1 versus a ratio of 1.2 to 1 in this DNA sequence). No strand bias for pyrimidine or purine mutations were observed in 12 PCR clones with multiple transition mutations. (Given one mutation, is the next mutation on the same strand similar?) There were six clones with mutations at only purine or pyrimidine bases on one strand, and six clones with mutations to both purine and pyrimidine bases on the same strand.

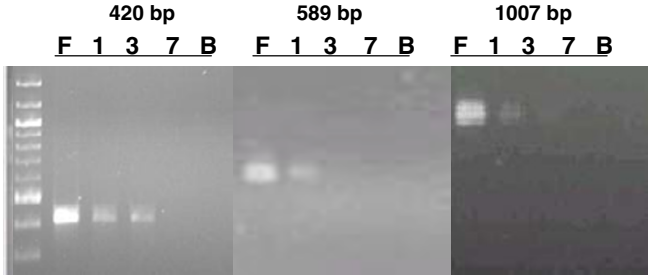

Figure 1
Composite figure of PCR products from DNA extracted from fresh tissue (F) or paraffin embedded tissues fixed in formalin for 1, 3 or 7 days. (B = water blank, the size marker is a 100 base pair ladder, with more intense 500 and 1000 base pair bands)

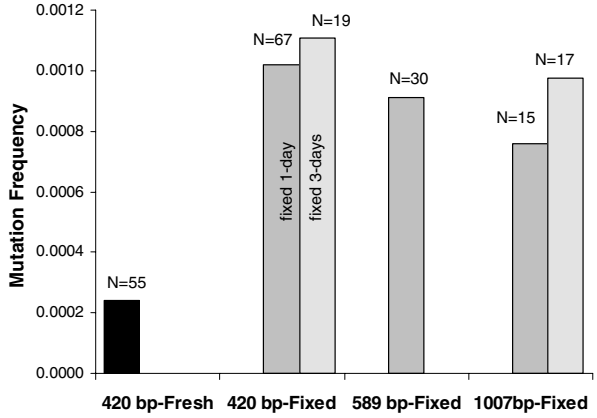

Figure 2
Mutation frequencies. (mutations per cloned sequenced base) of fixed DNA were 3- to 4-fold higher than fresh DNA. Mutation frequencies did not significantly change with PCR product size or fixation intervals. (N refers to numbers of sequenced PCR clones)

Discussion

The relative difficulty of producing PCR products with DNA extracted from fixed tissues is likely due to damage. Damage at even a single base may effectively destroy a template if it blocks polymerase elongation. However, DNA is a complex molecule and it has been difficult to characterize the types of damage caused by formalin fixation, especially because damage is likely to be randomly distributed or different between templates. Here we provide data through sequencing of multiple PCR product clones that fixed DNA likely contains randomly damaged purine and pyrimidine bases.

The greater mutation frequencies with fixed versus fresh DNA extracted from the same tissue are consistent with translesion synthesis during PCR across sites of base damage. Fresh DNA presumably lacks measurable DNA damage, and therefore mutations reflect polymerase fidelity, or the ability to match the correct base opposite a normal base. With damaged DNA, polymerase elongation either stalls at base damage (preventing PCR), or continues across sites of base damage through translesion synthesis (allowing PCR). Translesion by-pass is error prone because a damaged base is a nontemplate (abasic) or mistemplate. Although *Taq* DNA polymerase is not classified as a by-pass polymerase, it exhibits translesion synthesis with modified DNA templates [9-11], possibly because it lacks the 3' exonuclease activity typically found in A-family polymerases [7,12].

Recognition of translesion synthesis requires both a damaged or modified template, and the incorporation of a base opposite the damage. The higher frequencies of transition mutations with fixed DNA are consistent with widespread random base damage, predominantly at A:T base pairs in DNA extracted from formalin fixed, paraffin embedded human tissues. Double stranded DNA is resistant to formalin treatment [4], and higher mutation frequencies may reflect greater accessibility of formalin in tissues to more weakly H-bonded A:T base pairs. *Taq* DNA polymerase exhibits translesion synthesis during PCR, primarily creating *in vitro* transition mutations by inserting purines across from damaged pyrimidines, or pyrimidines across from damaged purine bases. Small deletions at simple short repeat sequences also provide evidence for bypass translesion synthesis, possibly by a dNTP-stabilized misalignment mechanism [13] with fixed DNA. Of note, initial efforts to perform translesion synthesis with *E. coli* error prone polymerases *Pol IV* or *Pol V* [5,6] prior to PCR did not improve subsequent product yields with fixed DNA templates (data not shown).

Other studies [14,15] have also noted higher frequencies of transition mutations when fixed specimens are examined by PCR. However, these studies observed higher mutation frequencies at G:C base pairs, suggesting the exact protocol for fixation, DNA extraction, and PCR may effect the mutations induced *in vitro* by fixation. The extent of damage in fixed DNA is likely greater than measured because some damage may be resistant to bypass, and *Taq* polymerase may also insert the correct base across from a damaged base. The exact nature of the base damage is unknown and would be difficult to discern because

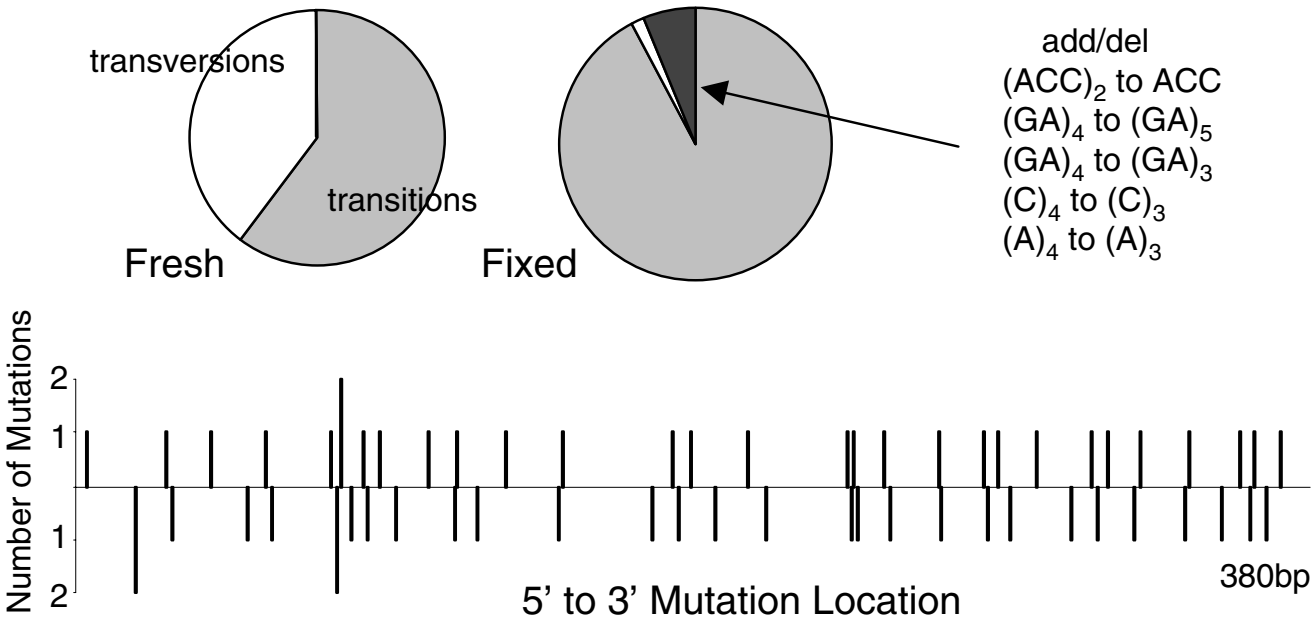

Figure 3
Mutation spectra. Differed between fresh (five mutations) and fixed (76 mutations) DNA, with marked increases in transition mutations, and additions or deletions at short repeat sequence. Mutations in the fixed DNA specimens appear randomly distributed. Graphed are mutation locations for the 380 base pair sequenced region within the 420 base pair PCR product (APC bases 4063 to 4442, GenBank Accession NM_000038.2).

only a minority of bases appear to have damage that can be successfully by-passed by translesion synthesis.

The conclusions of our study are limited by the small number of examined specimens, and primarily illustrate that DNA extracted from formalin fixed, paraffin embedded tissues may be damaged but still readable after *in vitro* translesion synthesis by *Taq* DNA polymerase. Translesion synthesis allows PCR of otherwise unreadable damaged DNA templates, but subsequent *in vitro* sequence changes from error-prone translesion synthesis add another potential complication to the genetic analysis of fixed tissues. *In vitro* artifacts from DNA tissue fixation damage and translesion synthesis should be invisible to most sequence studies because such errors are randomly distributed (non-clonal) and relatively infrequent (about one per 1,000 bases). However, such errors may become significant when small numbers of damaged templates are amplified or cloned PCR products are sequenced. The detection of multiple or non-clonal mutations from fixed tissues should raise the possibility of *in vitro* DNA damage and error-prone translesion synthesis.

Conclusions

Damaged bases are present in DNA extracted from formalin fixed tissues. These damaged bases are still "readable" but subject to error prone translesional interpretation/misinterpretation by *Taq* DNA polymerase during PCR. The sequence information originally present in the tissue is largely intact because bypass mutations are non-clonal and absolute numbers of mutations after cloning are low (about one per 1,000 bases). However, appropriate caution should be exercised when analyzing small numbers of templates or cloned PCR products derived from fixed tissue sources.

Competing interests

None declared.

Authors' contributions

NQ carried out the molecular studies and participated in the data analysis. MFG participated in the study design, data interpretation, and drafting of the manuscript. DS conceived of the study, and participated in its design, experiments, coordination, and drafted the manuscript. All authors read and approved the final manuscript.

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