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Research paper

Comparative reactivity of mismatched and unpaired bases in relation to their type and surroundings. Chemical cleavage of DNA mismatches in mutation detection analysis

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ABSTRACT

Systematic study of chemical reactivity of non-Watson–Crick base pairs depending on their type and microenvironment was performed on a model system that represents two sets of synthetic DNA duplexes with all types of mismatched and unmatched bases flanked by T·A or G·C pairs. Using comparative cleavage pattern analysis, we identified the main and additional target bases and performed quantitative study of the time course and efficacy of DNA modification caused by potassium permanganate or hydroxylamine. Potassium permanganate in combination with tetraethylammonium chloride was shown to induce DNA cleavage at all mismatched or bulged T residues, as well as at thymines of neighboring canonical pairs. Other mispaired (bulged) bases and thymine residues located on the second position from the mismatch site were not the targets for $KMnO_4$ attack. In contrast, hydroxylamine cleaved only heteroduplexes containing mismatched or unmatched C residues, and did not modify adjacent cytosines. However when G·C pairs flank bulged C residue, neighboring cytosines are also attacked by hydroxylamine due to defect migration. Chemical reactivity of target bases was shown to correlate strongly with the local disturbance of DNA double helix at mismatch or bulge site. With our model system, we were able to prove the absence of false-negative and false-positive results. Portion of heteroduplex reliably revealed in a mixture with corresponding homoduplex consists of 5% for bulge bases and "open" non-canonical pairs, and 10% for wobble base pairs giving minimal violations in DNA structure. This study provides a complete understanding of the principles of mutation detection methodology based on chemical cleavage of mismatches and clarifies the advantages and limitations of this approach in various biological and conformational studies of DNA.

1. Introduction

A study of the chemical behavior of non-Watson–Crick base pairs generated in vivo as replication errors is essential for understanding mismatch repair mechanisms. Additionally it provides a theoretical basis for numerous methods of point mutation and single nucleotide polymorphism screening. On the base of earlier investigations of DNA chemical properties [1], several chemicals were employed as structure-dependent modifying agents. Their action relies upon chemical recognition of mismatched base pairs in DNA duplex structure. Nucleobases at mismatched sites are partly flipped out of the stacking interactions due to disrupted hydrogen bonding and changed glycosidic bond angles, and therefore, become susceptible to chemical reactions. According to X-ray and NMR spectroscopy studies [2,3], the extent to which mismatched base is exposed to solution depends on the local geometry of DNA double helix that are intrinsically modulated by the type of mismatch and flanking bases. The interest in DNA duplex chemical behavior has increased due to development of highly sensitive methodology for random point mutation detection, such as Chemical Cleavage of Mismatches (CCM) [4,5]. This technique is based on consecutive denaturing and annealing of amplified normal and mutant DNA fragments, with a formation of mixture of mismatch-containing heteroduplexes and fully-matched homoduplexes.

Detection of point mutations as cancer markers can be of great value for diagnosis, choice of treatment strategy and for reliable prognosis. Cancer-associated point mutations can arise at the peculiar sites, so-called "hot spots", of oncogenes and practically at

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any site of conserved regions of suppressor genes. A wide range of reliable and efficient techniques has been developed for genetic testing of mutations at known physical locations [6–8]. However, there are no methods that detect all variations of random point mutations and that are sensitive enough to reveal mutant DNA in the presence of wild-type alleles. Thus, direct sequencing detects mutations when the portion of mutant DNA in a sample is at least 1/3rd [9–11]. This requirement is not always accomplished in analysis of DNA isolated from tumor tissues, because a random point mutation can arise only in one allele of a tumor cell, while the tumor stroma and infiltrating blood cells are of the wild-type genotype. In this case, the sensitivity of direct sequencing is insufficient. Another method applied for detection of random point mutations, namely Single-Strand Conformation Polymorphism (SSCP) analysis [10,12,13], is characterized by a high rate of false-negative results, since it reveals only those mutations that change the conformation of single-stranded DNA fragment.

The most promising CCM technique is capable of revealing unknown point mutations of random location and determining their position and type. Certain progress was achieved when potassium permanganate interacting to thymine in a combination with tetraethylammonium chloride (TEAC) was used instead of toxic osmium tetraoxide [14–16]. It is known that TEAC abolishes the preferential melting of T·A *versus* G·C base pairs due to high affinity to T·A pairs, hydrophobic side groups and low screening of the phosphate charges in DNA [14,17]. As this method requires purification of DNA after each reaction step, successful attempts have been made to attach biotinylated DNA samples onto streptavidin-coated magnetic beads for solid-phase chemical modification and cleavage reaction in order to overcome this problem [18]. CCM sensitivity was increased when differential fluorescent end labeling was used (Fluorescence Assisted Mismatch Analysis, FAMA) [19]. Different versions of CCM were successfully employed in the whole number of clinical assays [20–22], particularly, those used for detection of germline mutations in the genes determining some hereditary diseases.

Yet, the standard CCM protocol does not always produce a specific signal because of a high level of background DNA cleavage. In our previous study, we suggested that this could be explained by spontaneous formation of branched DNA structures, Holliday junctions, in solution of homologous linear duplexes [23,24]. Cross-point migration produces unpaired nucleotides that are the additional targets for chemical reagent. To minimize the quantity of branched structures, the heteroduplex formation procedure was optimized by varying the hybridization conditions, the composition of DNA duplex end regions, etc [25].

However, the lack of systematic investigation of mismatched base reactivity does not allow one to evaluate the sensitivity of CCM in relevance to mismatch type, orientation and flanking base pairs, as well as to control false-negative results. Considering chemical probing as an independent way to study the degree of local structural deviation at non-canonical base pair, Bhattacharyya and Lilley analyzed the dependence of cleavage efficiency on the type of mismatch using a set of plasmids with different nucleotide substitutions at a certain position [26]. However, their model constituted a mixture of two homo- and two heteroduplexes, which prevented the analysis of separate structure-specific cleavage reactions. In addition, the chemical modification protocol used at that time was not fully elaborated to allow the effective cleavage of all single DNA mismatches. Bui and others used for the first time the short synthetic oligonucleotides to form the DNA duplexes with different mismatches for spectroscopic study of potassium permanganate oxidation reaction [27]. However, the set of mismatches did not include all possible variants, and the influence of neighboring base pairs was not analyzed.

In this paper, we carried out the systematic study of comparative susceptibility to chemical modification for all possible DNA mismatches and bulge bases in different surroundings and described the cleavage patterns for each sample. To accomplish this, we used two families of 50-bp synthetic DNA duplexes with all kinds of mismatched and unmatched bases located in similar position and flanked by either T·A or G·C bas pairs. This model system was described in our earlier paper [28]. Potassium permanganate in a combination with tetraethylammonium chloride and hydroxylamine were used for base modification under developed optimal conditions. Designed model that represents the universal set of positive controls was applied to evaluate the CCM sensibility and specificity related to mismatch type and microenvironment. Comparative reactivity of different non-canonical base pairs was discussed in terms of correlation with the local disruption of double helix structure induced by certain mismatch.

2. Materials and methods

2.1. Oligonucleotide design and synthesis

Oligodeoxyribonucleotides were prepared by a solid-phase automated synthesis with subsequent biotin label incorporation at the 5′-end of the forward sequence and purified by electrophoresis on a denaturing polyacrylamide gel (Sintol, Russia). The following 50-mer oligonucleotides were used to obtain homo- and heteroduplexes containing a mismatch flanked by T·A base pairs.

The forward sequence (5′–3′) was

biotin-CGGCGGCCTTGTGGTAGTTGGACA**TXT**AGGCGTAGGCAAGAG TCGCCCGG, where X is G (1), C (2), A (3), T (4) or deletion (5); the numbers of oligonucleotides are shown in parentheses.

The reverse sequence (5′–3′) was

CCGGGCGACTCTTGCCTACGCCT**AYA**TGTCCAACTACCACAAGGCCGC CG, where Y is C (6), G (7), A (8), T (9) or deletion (10).

Another library of heteroduplexes with G·C pairs flanking the single base mismatch was formed by oligonucleotides 11–20.

The forward sequence (5′–3′) was

biotin-CGGCGGCCTTGTGGTAGTTGGACA**GXG**AGGCGTAGGCAAGA GTCGCCCGG, where X is G (11), C (12), A (13), T (14) or deletion (15); the reverse sequence (5′–3′) was CCGGGCGACTCTTGCCTACGCCT**C YC**TGTCCAACTACCACAAGGCCGCCG, where Y is C (16), G (17), A (18), T (19) or deletion (20).

The purity of oligonucleotides was verified by gel electrophoresis analysis, using ^{32}P-labeled probes.

2.2. DNA duplex formation

Two libraries of heteroduplexes containing all possible mismatches and bulge bases in both orientations within the double helix were prepared by pairwise hybridization of twenty 50-mer oligonucleotides of forward and reverse sequences differing only in one central nucleotide unit (Table 1). Equal molar amounts of appropriate oligonucleotides were mixed to the final concentration of 10 ng/μl in 1 M NaCl, 2 mM EDTA, 6 mM Tris–HCl (Sigma), pH 7.5. Oligonucleotide mixtures were heated to 95 °C for 2 min and then cooled down to 42 °C by stepwise reduction of temperature by 2 °C every 30 s. The mixtures were maintained at 42 °C for 60 min and finally brought back to room temperature.

2.3. Immobilization of DNA duplexes on Dynabeads M-280

Biotinylated DNA duplexes (100 ng) were immobilized on streptavidin-coated Dynabeads M-280 magnetic beads (Dynal Biotech) with regard to 1 mg of beads binds 5 pmol of double-stranded DNA. Previously, the suspension of magnetic beads was