HDCR: A novel method for the diagnostic detection of methylated DNA for blood based cancer diagnostics

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ABSTRACT

Release of hypermethylated DNA from tumour cells affords the opportunity to develop an early diagnostic for cancer by DNA isolated from blood or other bodily fluids. However, using standard methylation-specific PCR, DNA frist needs to be treated with bisulfite and sensitivity can be limited due to mispriming . Once formed, unwanted products amplify efficiently and can prevent detection of the target. To overcome this we have developed a system which does not require bisulfite treatment of DNA and in which selectivity is maintained throughout the amplification.

Keywords: diagnostic, blood-based, PCR, cancer

INTRODUCTION

Methylation of cytosines at CpG sites in mammalian DNA, is a key component of epigenetic programming of mammalian development. Different cell types and tissues have characteristic patterns of methylation that are maintained through cell division. As well as frequent mutations and rearrangements of the genome, genomes of cancer cells are characterised byextensive aberrant methylation of DNA. Increasing data indicates that DNA methylation and other epigenetic changes are also associated with cardiovascular disease, rheumatoid arthritis, multiple sclerosis and other diseases.

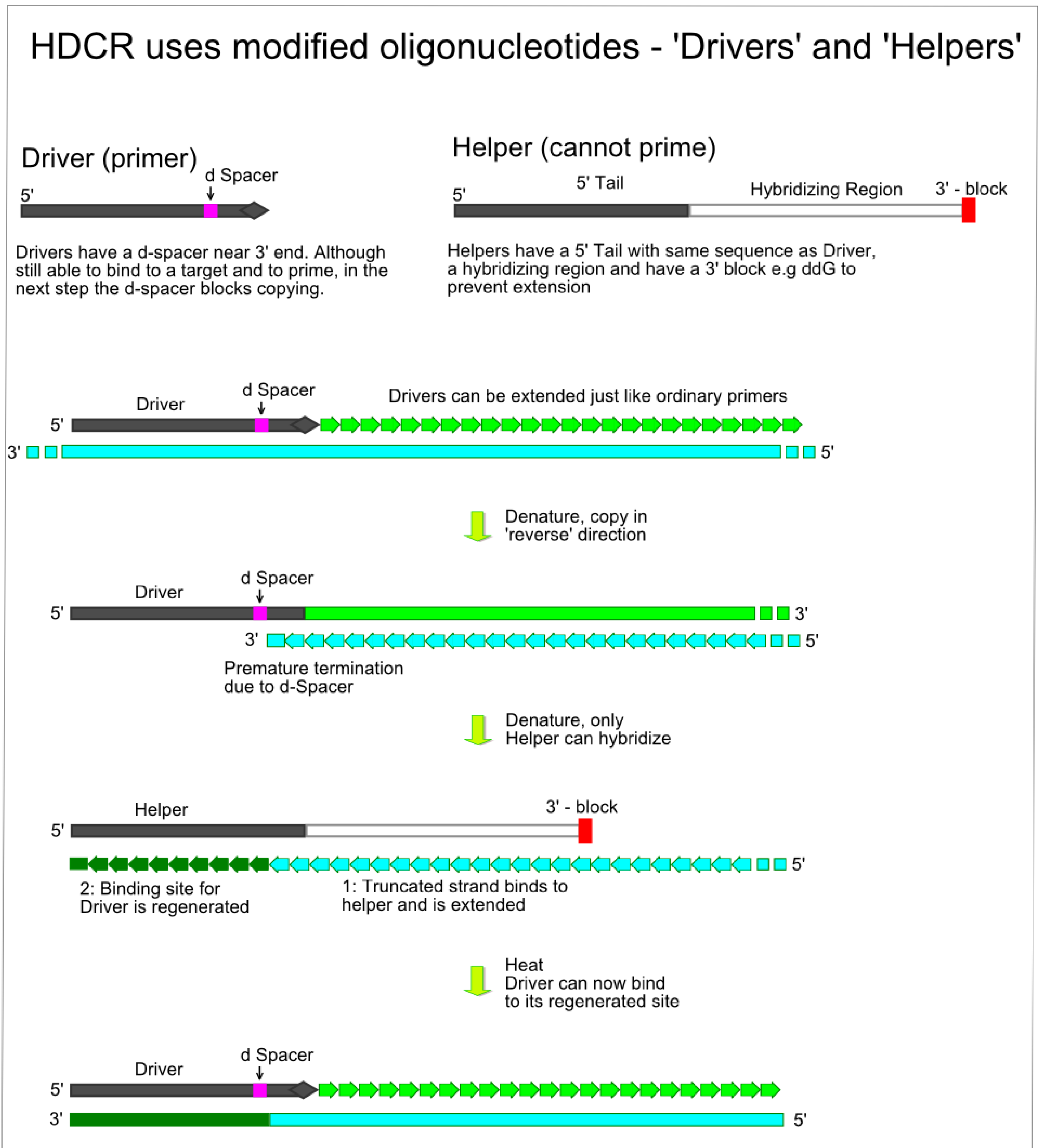
Promoter regions of many genes, typically “CpG islands” frequently become methylated in different cancer types and this methylation is associated with silencing of gene expression. The frequent methylation of specific genes in a high fraction of different cancers has led to development of assays for early cancer detection. Cancer cells and/or DNA from cancer cells are released from primary cancers and cancer-derived DNA can be found in blood and other accessible fluids such as blood, sputum and urine. Examples of genes that are methylated in a high fraction of particular cancers are GSTP1 in prostate cancer, *SHOX2* in lung cancer and *SEPT9* and *VIM1* in bowel cancer. Assays are under development or have been commercialized for these DNA methylation biomarkers.

The potential prognostic or predictive use of DNA methylation biomarkers and their use in classification and stratification are also now being actively explored. Methylation of the DLX2 gene has been associated with risk of progression in breast and prostate cancers, while methylation of the *MGMT* DNA repair gene is being used to guide alkylating agent chemotherapy for patients with gliomas. Current assays for detection and quantifying gene-specific DNA methylation are based on PCR following treatment of DNA with sodium bisulfite. This procedure is time consuming and leads to significant DNA loss.

Recently, a number of restriction enzymes that cut at specific sequences only when they are methylated have been identified and are available commercially. For a number of these their cutting specificity includes methylated CpG sites as found in mammalian DNA. Examples are GlaI and PcsI (Sibenzyme) and MspJ1, LpnP1 and FspEI (New England Biolabs). We have combined the use of such enzymes with a novel amplification method that allows specific amplification of rare methylated DNA fragments in a high background of unmethylated sequences and that avoids the need of bisulfite conversion. The methylation-dependent restriction enzyme is used to selectively cut methylated DNA, producing short fragments of the target sequence. Then targeted fragments are tagged using specially designed “Helper” oligonucleotides that are also used to maintain selection in subsequent amplification cycles in a process called ‘Helper-Dependent Chain Reaction (HDCR). The process uses disabled primers called ‘Drivers’ that can only prime on each cycle if the Helpers recognize specific sequences within the target amplicon. In this way, selection for the sequence of interest is maintained throughout the amplification, preventing amplification of unwanted sequences. This is illustrated in Figure 1.

We have shown how the method can be applied to meSeptin 9, and other candidate biomarkers, GRASP and IRF4, for early diagnosis of colorectal cancer.[1] The digestion and subsequent amplification can all be done in a single tube. A detection sensitivity of 0.1% methylated DNA in a background of unmethylated DNA was achieved, which was similar to the well-established assays based on bisulfite-treated DNA.

Figure 1 – The principles of the technology for Helper dependent PCR



METHODS

DNAs and oligonucleotides

DNA from the leukemic cell line K562 was obtainedfrom Promega. CpGenome Universal Methylated DNA is fully CpG-methylated human male genomic DNA is available from Millipore (http://www.millipore.com/ catalogue/item/ s7821). Tumor samples were obtained from patients undergoing surgery at Flinders Medical Centre (Adelaide, Australia). Consent was obtained prior to surgery—Human Research Ethics Committee approval RGH 09/04. Tissue was disrupted using the Retsch TissueLyser (Qiagen) and DNA from cancer and matched normal tissue isolated using a Wizard_ Genomic DNA purification Kit (Promega). Oligonucleotide sequences are available in reference 1. All oligonucleotides were dissolved in TEX buffer (10mM Tris–HCl, 0.1mM EDTA, 0.01% Triton X-100).The helper oligonucleotides (denoted as 'helper/s' from hereon) were purchased from Biosearch Technologies (Novato, CA, USA) or from GeneWorks (Adelaide, Australia). Other oligonucleotides were purchased from GeneWorks or from Sigma-Aldrich (Sydney, Australia).

GlaI digestion

The restriction enzyme GlaI was purchased from Sibenzyme (http://www.sibenzyme.com/info627.php). DNAs were pre-cut with GlaI by digesting 1 µg in 50 µL 'SE Buffer GlaI' (10mM Tris–HCl, pH 8.5 at 25_C; 5mM MgCl2; 10mM NaCl; 1mM 2-mercaptoethanol) plus 16 units of GlaI for 2 h at 30°C followed by 15 min at 70ºC. Reactions were stopped by addition of 5 µl of 50mM EDTA and 145 ml of TEX buffer Digested DNAs were stored at -20ºC.

Helper-dependent chain reaction

10 µL HDCR reactions were done in a Corbett Rotor-Gene 3000 machine with a 72-tube rotor and. Conditions for hot start PCR are 20mM Tris–HCl (pH 8.4), 50mM KCl, 7.5mM MgCl2, 0.2mM dNTPs, oligonucleotides, 1/50 000 dilution of SYBR Green, 0.01mM dithiothreitol, 0.1% Triton X-100 and 0.04 U/ml Platinum Taq Polymerase. When restriction digests are done in the PCR tube GlaI is included at 0.04 units/µl .Suitable cycling conditions for HDCR for 10 µl volumes and the Rotor-Gene 3000 using pre-cut DNA samples are five cycles (90ºC 15 s, 55ºC 20 s, 76ºC 5 s, 67ºC 20s) followed by second stage of up to 85 cycles (90ºC 15s— read HEX channel, 67_C 40ºs, 76ºC 5 s, 67ºC 20 s—read FAM/SYBR Green channel). When GlaI digestion was done in the PCR mix, an initial incubation of 10 min at 30ºC was carried out. The inclusion of SYBR Green allows a melt curve analysis to be carried out after the amplification. Addition of a low level of Triton X-100 in some situations improves the SYBR Green signal; possibly by preventing loss of the hydrophobic SYBR Green to surfaces. The dithiothreitol appears in our experience to be needed to stabilize some batches of Platinum Taq Polymerase, especially when high denaturation temperatures are used. With improved design of Helper oligonucleotides, more efficient reactions and much shorter cycling times have now been obtained(1). .

RESULTS

We have developed a novel method that avoids the need for bisulfite conversion of DNA and provides specific amplification of rare methylated DNA fragments in a high background of unmethylated sequences that. A methylation-dependent restriction enzyme such as GlaI or LpnP1 is used to selectively cut methylated DNA. Following digestion targeted fragments are tagged using specially designed 'helper' oligonucleotides (Fig. 1). These Helpers are also used to maintain selection in subsequent amplification cycles in a process called 'helper-dependent chain reaction'. HDCR uses disabled primers called 'drivers' that can only prime on each cycle if the helpers recognize specific sequences within the target amplicon. Thus, selection for the sequence of interest is maintained throughout the amplification and

HDCR of the hypermethylated Septin 9 gene in clinical samples.

To evaluate the use of HDCR on clinical samples we determined the level of hypermethylated Septin 9 gene in CRC and matched normal tissues from 25 cancer patients (four Stage A, nine Stage B, eight Stage C, one Stage D and four unknown stage). The level of methylation was determined relative to that of fully methylated CpGenome DNA. For quantification, all samples were diluted such that 2 ng of each was assayed with a standard curve ranging from 100 pg to 2 ng of fully methylated CpGenome DNA (mixed with K562 DNA in which Septin 9 is unmethylated to make a total amount of 2 ng, where necessary). The standards were cut by GlaI in the initial incubation of the PCR tubes at 30ºC, along with the test samples. A clear separation of cancer and normal tissue

DNA methylation results. In considering individual samples, HDCR could detect hypermethylation in the Septin 9 gene to a level of >5% in 22/25 (88%) patients. For all but two of the subjects, methylation of the Septin 9 gene was substantially higher in the cancer than matched normal DNA. For these two patients, methylation levels were similar in matched normal tissue. This could indicate methylation variability within normal tissue, methylation in the zone of pre-neoplastic tissue in which the cancers arose, or that the normal tissue was contaminated with neoplastic cells. We compared the Septin 9 HDCR assay with the heavy methyl assay that is now in clinical use. A significant correlation between the two methods was shown (P=0.0003, Spearman's rho: 0.6176, Graph Pad Prism, version 5).Despite the generally good correlation, in some patients the methylation level determined by HDCR and heavy methyl assays differed substantially; these differences may reflect the pattern of CpG site methylation in the individual patients as selection in the two assays is based on different CpG sites in the same region

CONCLUSIONS

HDCR has been demonstrated to be able to detect low levels of methylated DNA in complex mixtures.

The design of HDCR makes it readily amenable to multiplexing, since a mix of gene-specific helpers sharing a common 'tagging' sequence could be used in combination with a single driver at each end. This would be ideal for use in detection of a panel of biomarkers in clinical samples. We have demonstrated the feasibility of a triplex reaction [1].

Because selection for the targeted sequence can be maintained throughout amplification, HDCR should be considered for any applications in which non-specific amplification is a problem. False negatives can be a problem when only very low levels of a target are present in a vast excess of normal DNA. Use of HDCR might overcome this because selection can be maintained throughout the entire amplification, thus improving overall specificity.

While described here in the context of detection of hypermethylated genes, HDCR has features that can be adapted for use in a wide range of applications. We envisage that through appropriate target-specific design of the driver and helper oligonucleotides, HDCR could be adapted to detect for example

- hypomethylated sequences,
- gene mutations,
- microsatellite instability (repeats of different length) and
- microRNAs

in clinical samples.

Patent WO2009043112

REFERENCES

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