Development of Electrochemical DNA Detection Methods to Measure Circulating Tumour DNA for Enhanced Diagnosis and Monitoring of Cancer

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Abstract: Liquid biopsies are becoming an increasingly important potential replacement for existing biopsy procedures which can be invasive, painful, and compromised by tumour heterogeneity. This paper reports a simple electrochemical approach tailored towards a point of care cancer detection and treatment monitoring from biofluids using a label-free detection strategy. The mutations under test were the KRAS G12D and G13D mutations which are both important in the development and progression of many human cancers and whose presence correlates with poor outcomes. These common circulating tumour markers were investigated in clinical samples and amplified by standard and specialist PCR methodologies for subsequent electrochemical detection. Following pre-treatment of the sensor to give a clean surface, DNA probes developed specifically for detection of the KRAS G12D and G13D mutations were immobilized onto low-cost carbon electrodes using diazonium chemistry and EDC/NHS coupling. Following the functionalisation of the sensor, it was possible to sensitively and specifically detect mutant KRAS G13D PCR product against a background of wild type KRAS DNA from the representative cancer sample. Our findings give rise to the basis of a simple and very low-cost system for measuring ctDNA biomarkers in patient samples. The current time to result of the system is 4.5 h with considerable scope for optimisation and already compares favourably to the UK National Health Service biopsy service where patients can wait weeks for their result. The paper will report the technical developments we have made in the production of consistent carbon surfaces for functionalisation, assay performance data for KRAS G13D, detection of PCR amplicons under ambient conditions.

Keywords: electrochemical; DNA biosensors; KRAS; liquid biopsy; cancer point of care diagnostic tests

1. Introduction

Cancer is a genetic disease by nature, caused by mutations in certain genes thereby resulting in cellular malfunction [1]. Imaging tests can sometimes be inconclusive and generally do not provide information on the stage or type of cancer, so further biopsy will be needed [2]. Collecting multiple biopsies from different regions of a primary tumour and associated metastasis is invasive and may pose serious medical risks [3]. The time point at which a tumour manifests clinical symptoms often correlates with the later stages of progression (e.g., Phases III & IV) where surgery and therapy are less effective because of e.g., metastasis or tumour cells acquiring a critical mass of mutations [4].
Furthermore, for some patients, surgical biopsy procedures are not possible therefore liquid biopsies that detect the presence of tumour DNA in blood, hold promise as a non-invasive alternative.

Short fragments of cell-free DNA (cfDNA) are detectable in most body fluids including blood. In cancer patients, a fraction of cfDNA called circulating tumour DNA (ctDNA) can be found, which originates from tumours and may carry the same mutations and genetic alterations as those present in the primary tumour [5]. Although circulating tumour cells (CTCs) that have been shed into the vasculature of a primary tumour are carried around the body in circulation, they are present at levels of around 10 cells/mL of blood, indicating that only very low concentrations are present in clinical samples. Compared to ctDNA, CTCs are rare in peripheral blood and are difficult to separate from other cells adding further credibility to the use of ctDNA for liquid biopsy applications. Although the mechanism of ctDNA release from tumour cells is poorly understood [6], it is thought to be released in small quantities following apoptosis or necrosis. ctDNA usually comprises 0.01–1% of the circulating free DNA in blood [7] and it is important to note that this can be shed as both single stranded and double stranded DNA [8]. ctDNA can currently be detected in blood and other body fluids like lymph, urine and stool [9]. Due to the small fraction of ctDNA masked in by large background levels of wildtype cell-free DNA, highly sensitive amplification reactions such as polymerase chain reaction (PCR) will need to be employed to achieve discrimination. It is possible that a PoC measurement of circulating tumour DNA (ctDNA) may present a more practical means of detecting the presence of a tumour as well as capturing tumour heterogeneity, evaluating response to treatment and monitoring disease recurrence [9,10].

V-Ki-ras Kirsten rat sarcoma (KRAS) is a member of the RAS family of proteins which are a part of at least six signalling pathways in a healthy human. KRAS is the most frequently mutated protein in human tumours with KRAS G12D and G13D being specific variants found across many tumour types including pancreatic, colorectal, non-small cell lung, and ovarian cancer [11]. KRAS activated mutations drive cancer initiation, progression, and drug resistance, directly leading to nearly a million deaths per year. KRAS mutations take place in approximately 90% of pancreatic cancers [12], 30% of lung cancers [13], 60% of thyroid cancers, and 43% of colorectal cancers [14]. The topic of liquid biopsies is one such area where innovations in biomarker detection will enhance clinical outcomes for patients [15].

Electrochemical DNA biosensors represent an exciting approach to the fast, low-cost detection of clinically important biomarkers [16,17] at the point of care [18]. Electrochemical biosensors are used to directly convert a biological binding event to an electronic signal [19]. A range of electrode materials and electrochemical measurement approaches have been employed for sensitive measurements (cyclic voltammetry [20], differential pulse voltammetry [21], square wave voltammetry [22], and electrochemical impedance spectroscopy [23]). The potential of electrochemical biosensors, once matured as a technology to provide efficient clinical workflows is immense. In DNA biosensing, a change in signal is obtained when recognition and hybridisation of two opposing strands of DNA occur as a result of their base-pair complementarity. A double stranded DNA sequence with tumour-specific mutations can indicate the diagnosis of a specific cancer [24].

As the concentration of ctDNA increases when patients have advanced cancers, achieving high sensitivity for the DNA sensor is important for the early detection of disease, and developing tailored therapies. Carbon electrodes are chemically inert, particularly at negative potential ranges in all media making them highly suitable electrode sensors for electroanalytical chemistry and giving them an advantage over metal electrodes[25]. Screen Printed Carbon electrodes (SPCEs) are homogenous, simple, sensitive, cost-effective (~£2 each) and disposable making them preferable for rapid electrochemical analyses and suitable as electrodes for characterizing the processes we employed, which were Cyclic Voltammetry (CV) and Differential Pulse Voltammetry, two routinely used electrochemical measurement techniques.

CV and DPV are common analytical techniques that supply information on electron transfer reaction kinetics of any combined chemical reaction [26]. In both techniques, a potential waveform is applied to the working electrode (WE). The peak current obtained is directly influenced by hybridisation between target and immobilised probe DNA strands [27]. Both DPV and CV are useful
for studying electro-activity and characterising the reductive and oxidative properties of compounds in solution. This work presents a KRAS G12D and G13D DNA oligonucleotide probe modified sensor array that can accurately detect mutant KRAS amplicons and therefore forms the basis of a system for the accurate detection of ctDNA in patient samples and monitoring of response during treatment. This was achieved by amplifying mutant DNA isolated from a human cancer cell line recovered from clinical samples, using electrochemical techniques and SPCEs to detect a clinically relevant mutation, comparing the signal change from DNA hybridisation experiments involving wildtype KRAS and amplified KRAS mutant samples, varying concentration of amplified products to determine concentration effects and establishing a limit of detection for the DNA amplification reaction. In this study, both DPV and CV were used depending on whether electrodes needed to be cleaned, electrografted, or characterised during sensor measurement. Different voltage and scan ranges were selected for the measurements carried out. Due to the simplicity of the approach presented herein and the choice of steps employed in the assay, the system can be very easily automated and integrated into a final device capable of fast and seamless clinical measurements.

2. Materials and Methods

2.1. Reagents

Droplet digital PCR (ddPCR) assays, Supermix for probes, DG8TM cartridges and Droplet Generation Oil were obtained from BioRad Laboratories Ltd, UK. Deionized water, sodium chloride, phosphate buffered saline (PBS), sodium nitrate, 4-aminobenzoic acid, hydrochloric acid, ethanolamine, 2-((N-morpholino)ethanesulfonic acid (MES), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), potassium ferricyanide, potassium chloride, and potassium ferrocyanide were all purchased from Sigma-Aldrich, UK. 250 units HotStarTaq Plus and dNTP Mix, PCR Grade (200 μL) were purchased from Qiagen, UK. Phusion direct PCR kit was purchased from Thermo Scientific.

2.2. Electrochemical Setup

Screen printed multi carbon electrodes (DRP 8W110) as shown in Figure 1A below was obtained from DropSens (Oviedo, Spain) with chip dimensions of 50 × 27 × 1 mm (L × W × D). The chip contained eight carbon working electrodes with diameters of 2.95 mm with a carbon counter and silver reference electrode. The screen-printed fabrication process is specified by the manufacturers (Dropsens, 2019).

![Figure 1](image)

Figure 1. (A) Image of screen printed electrode array employed (8 × working electrodes with common Ag reference and carbon counter electrodes along with schematic showing DNA functionalisation and DNA target binding (B) DPV results showing repeated cycling of the 8 working electrodes prior to modification and DNA introduction using the same current and voltage inputs.
2.3. Electrochemical Measurements and Surface Functionalisation

DNA hybridisation experiments were performed using a covalently attached layer of single stranded DNA probes. The surface functionalisation protocol is illustrated in Figure 1A. To prepare the surface of the carbon electrodes for DNA probe attachment it was necessary to first use a surface pretreatment by applying 1.4 V for 1 min in 0.5 M acetate buffer solution (ABS) containing 20 mM NaCl 8 (pH 4.8) via CV. Next, 2 mM NaN0₂ solution with 2 mM 4-amino benzoic acid was prepared in 0.5 M HCl and stirred for about 5 min at room temperature to produce a diazonium compound. The activated diazonium solution was then scanned using CV from +0.4 to –0.6 V at a scan rate of 100 mV/s followed by a wash with DI water. The resulting 4-carboxyphenyl (AP) film was activated on the electrode surface with 100 mM EDC and 20 mM NHS in 100 mM MES buffer (pH 5.0) for 60 min to form an ester that allowed efficient conjugation to the amine-modified ssDNA probe. Ferricyanide buffer (5 µM) was used to characterise the sensor surface. Unless specifically stated, all the reported steps and measurements were carried out at room temperature.

2.4. Genomic DNA Sample Preparation, DNA Probe Design and Sample Amplification

For assay development, copies of the KRAS pG13D mutant and wild type DNA were amplified from genomic DNA (gDNA) isolated from SK-UT-1 cells. Levels of both mutant and wild type DNA were determined using ddPCR assays (KRAS WT for p.G13D c.35G>A, assay ID DHsaCP2000002) in combination with a QX200TM Droplet DigitalTM PCR system (Biorad Laboratories Ltd., UK) following the manufacturer’s instructions. Briefly, 5-10ng of gDNA isolated from SK-UT-1 cells- was mixed with ddPCR Supermix for probes (N0 dUTP) and fluorescein amidite (FAM)-labelled KRAS p.G13D primers/probe and hexachloro-fluorescein 9 (HEX)-labelled KRAS WT primers/probes, in the presence of restriction enzyme and in a volume of 20 µL. Reaction samples were loaded onto a DG8TM cartridge with 70 µL of Droplet Generation Oil for Probes according to the Droplet Generator Instruction Manual (Biorad Laboratories Ltd., UK). PCR cycling conditions for the generated droplets were as follows: initial enzyme activation at 95 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 30 s, and annealing/extension at 55 °C for 1 min, after which ended with a final enzyme deactivation at 98 °C for 10 min. Data acquisition after thermal cycling was performed using the QX200 Droplet Reader and the QuantaSoft Software (Biorad Laboratories Ltd.).

The design of PCR primers and probes used in this study was based on the published sequence of KRAS pG12D and pG13D under accession number NC_000012.12 (NCBI, 2019). Amine-modified synthetic oligonucleotides (KRAS G13D) designed as shown in Table 1 below with a concentration of 200 µM was obtained from Sigma-Aldrich, UK, and stored at −80 °C prior to aliquoting for use as probes. A wild type probe (without the single base mutation) was also designed for use as a negative control. DNA probe stocks were diluted to a concentration of 2 µM in 0.1 × PBS prior to immobilisation. Primer-BLAST software was used to design the PCR primers employed in this study. The forward primers had a GC content of 55% while the reverse primer had a GC content of 39.13% with an estimated product length of 88 with low self-complementarity.

<table>
<thead>
<tr>
<th>KRAS G13D Probe and Primer Sequences</th>
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<tbody>
<tr>
<td>23 bases Wild type hybridisation Probe</td>
</tr>
<tr>
<td>23 bases Mutant hybridisation Probe</td>
</tr>
<tr>
<td>Forward Primer (Wild type)</td>
</tr>
<tr>
<td>Forward Primer (Mutant)</td>
</tr>
<tr>
<td>PCR Probe (Mutant)</td>
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<td>Reverse Primer</td>
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For PCR amplification, samples of extracted wild type and KRAS G13D mutated DNA was amplified using the phusion direct PCR kit following the protocol and reaction setup guide outlined by Thermo Scientific, UK. Phusion blood II DNA polymerase (1 µL), 2x PCR Buffer (25 µL), 50 mM
EDTA (2.5 µL), 50 mM MgCl₂ solution (1.5 µL), and 100% DMSO (2.5 µL) were all included in the reaction mix and dispensed into appropriate PCR tubes. 1 µL Template DNA, 5 µL forward and reverse primers, and 10 µL ultrapure water was added to the master PCR tube containing the reaction mix and the thermal cycler was programmed to start with an initial heat-activation step at 98 °C for 300 s. Temperatures specifications for denaturing, annealing, and extending were set at 90 s for 94 °C, 65 °C, and 72 °C respectively. A final extension for 60 s at 72 °C was set and PCR conditions were set for 37 cycles. PCR amplification of wild type and KRAS G13D samples was performed using the minipcr thermal cycler [28] and amplicons concentrations were confirmed using the Qubit 4 fluorometer and dsDNA broad range quantification assay [1].
3. Results and Discussion

3.1. Assay Workflow and Development

The use of a SPCE with multiple working electrodes allows each electrode to be individually modified whilst rapidly carrying out simultaneous measurement of peak currents and charge transfer resistance. The electrode functionalisation process is summarised in Figure 1A. Electrografting using in-situ generated diazonium cations is important for modifying the surface of the SPCE by allowing the formation of covalent bonds between the carbon surface and organic films. EDC is an established zero-length cross-linking agent that has been employed in coupling carboxyl groups to primary amines in varied applications [29]. One of the main benefits of EDC coupling is its water solubility that allows direct bioconjugation without prior organic solvent dissolution. To increase the stability of the active ester, NHS was introduced to modify the amine-reactive chemical substance by converting it to an active NHS ester, thus maximising the efficiency of the EDC-mediated coupling reactions. The reproducibility of this hybridisation sensor was explored by simultaneously analysing all 8 Wells from the multielectrode after the surface modification steps. Figure 1B shows a high level of consistency in the peak height, potential, and width. The multiplexed analysis we employed greatly reduced analysis time because of high throughput of samples and minimized reagent consumption. After introducing the probe solution to the surface of the modified electrodes, the remaining active groups on the electrode were blocked using ethanolamine to produce a consistent sensing layer in order to enable DNA specificity and stability in terms of the DNA binding response.

3.2. DNA Sensor Hybridisation Specificity

Having achieved a consistent behaviour of modified electrodes on the same chip, the next step was to test the assay’s response to incubation in a representative KRAS sample using the designed probes. We explored the ability of the probe modified electrodes to discriminate between G13D mutant and wild type KRAS sequences in representative clinical samples. To investigate specificity levels and gain an initial impression of assay sensitivity, a series of electrodes were functionalised with KRAS G13D mutant and wild type probe sequences. The results of these experiments are summarised in Figure 2, which shows the percentage change in the DPV peak current following target hybridisation. For macroscale electrodes functionalised with biological molecules such as DNA or antibodies the expectation is that differential pulse voltammetric peak currents will reduce upon target hybridisation. It has been observed that these effects can be reversed when micro or nanoscale electrodes are employed [17,30], but for this study, the electrodes employed were comfortably on the macro scale (diameter = 2.95 mm). Therefore, we would expect a reduction in peak current when specific DNA hybridisation had taken place and this was found to be the case for lower concentrations of DNA (pico to low nanomolar concentrations). For nanomolar (>10 nM)and micromolar concentrations, an increase in the peak current following hybridisation was consistently observed (and has also been observed in other unpublished data from our lab involving SPCEs) for carbon electrodes which is likely explained by the high surface density of hybridised DNA amplicons changing the interfacial properties of the electrode and therefore altering the electrochemical response. The underlying physical mechanism of this effect is actively under investigation. Figure 2A shows that when mutant and wild type oligonucleotide probe sequences functionalised SPCEs were incubated in a representative sample containing the G13D mutation, there was hybridisation in both cases, the signal change was greater for the wild type because of the high background of wildtype DNA and the comparatively low fraction of mutated KRAS G13D present in the representative clinical sample. Similar behaviour was observed for KRAS G12D probe functionalised electrodes for a representative sample for that particular mutation, showing the wild type KRAS DNA hybridised strongly to the nucleic acid modified carbon surfaces. As a result of these findings and the inability to electrochemically discriminate between positive and negative samples owing to the strong influence of background DNA in the sample, DNA amplification strategies were
developed and tested in order to ensure the production of unequivocal detection of ctDNA mutations.

![Figure 2](image)

**Figure 2.** (A) DPV percentage signal change in response to mutant and wild type probes hybridized with genomic KRAS G13D ssDNA. (B,C) Electrochemical signal change in response to incubated KRAS G12D ssDNA hybridized with mutant and WT oligonucleotides respectively.

### 3.3. KRAS G13D Amplification

Primers used for the PCR amplification were tailored to selectively amplify the mutant target from a pool of mutant and wild type sequences in a sample by varying the single nucleotide responsible for the mutation in the primer sequence. Adopting this approach allowed us to effectively enrich the number of mutated DNA sequences in the sample without amplifying the wild type, in order to produce a signal change above the background signal generated by KRAS wild type DNA non-specifically associating with the oligonucleotide probe sequences for KRAS G13D.

In selecting the approach reported here, ctDNA detection could potentially be coupled to a DNA amplification reaction because it allows the possibility of developing a multiplexed panel of DNA sequences on a single chip, meaning that commonly mutated genes could all be identified in parallel (e.g., KRAS, TP53, BRCA1&2, IDH-1 etc.). This approach of developing biomarker “panels” is thought to be one of the key advantages of the approach [31].

When the wild type probe sequence modified electrodes were hybridised with KRAS G13D amplicons, alterations in the peak current were not observed indicating no significant hybridisation. The mutant amplicons when incubated with mutant probe modified electrodes gave rise to a very significant signal change (~350%) indicating hybridisation with highly concentrated DNA samples (nano-micromolar concentration ranges) because of the strong positive signal change. Subsequent UV-vis measurements on the amplicon confirmed this to be the case with estimated amplicons concentrations being in the range of 250–500 µM. These findings were highly satisfying, i.e., that the surface tethered KRAS G13D mutant probe sequence could in fact discriminate between the mutant and wild type samples based on the presence or absence of PCR amplicons for KRAS G13D high sensitivity. This in fact represented a sort of double specificity for the PCR-based assay because the primer design had already been shown to specifically amplify the mutated sequence so coupling in the specificity of the electrochemical probe sequence meant that the assay would be able to successfully discriminate mutant amplicons from the sample. Having established the specificity of
the assay and the nature of the electrochemical change, the next step involved verifying the sensitivity of the assay and dose response effects for the KRAS G13D mutant PCR product.

3.4. Concentration dose Response

After establishing PCR primer specificity, ssDNA probe specificity and electrochemical signal changes of the correct direction and magnitude, it was important to investigate dose response effects. In these experiments, non-amplified and amplified samples were diluted and a dose response curve was constructed (see Figure 2A,B). For the unamplified sample, the lowest concentration (0.85 ng/µL) showed the lowest reduction in oxidative peak current post hybridisation with signal change increasing as sample concentration increased. The issue here, however, is the specificity of the probe-target interaction (as shown earlier) and the relatively small signal change brought about by incubation with unamplified samples. Owing to the fact that these were relatively low concentrations of DNA, leading to limited hybridisation, the signal changes were negative in direction i.e., decreases in DPV peak current. On the other hand, the amplified sample produced a dose-response curve with higher signal changes which were positive in direction due to the specific enrichment of the mutant sequence concentration with smaller standard deviations because of the hybridisation of strands of similar lengths (the unamplified samples contains heterogenous strand lengths because it is unprocessed DNA) and in effect the full fraction of cfDNA from a patient. Achieving good sensitivity is very important as the concentration of circulating free DNA released by tumour cells is usually in proportion to the stage of cancer [32]. As circulating nucleic acids are present in blood at ng/mL levels, which based on the fragment length is analogous to a picomolar concentration, a minimum of femtomolar sensitivity will be beneficial for detection of tumour-specific sequences [15]. Many published biosensor studies achieve such sensitivity levels through the use of exotic electrode modifications, typically involving the production of electrodes modified with graphene, nanoparticles, carbon nanotubes etc. In our case, we have opted to keep the electrode substrate, low-cost, and easy to produce and couple to a PCR reaction to achieve the desired sensitivity and specificity. Whilst our approach leads to a trade-off in terms of time to result, it ensures specific amplification and sensitive and specific hybridisation signals giving confidence in the result whilst achieving an overall time to result which is a significant improvement over the current clinical practice. The ctDNA concentration response shown in Figure 3 shows a clear dose response effect which predicts that an increase in ctDNA, per unit concentration, will result in larger electrochemical signal response in the positive direction (i.e., increasing DPV peak current). Since levels of ctDNA are strongly correlated with tumour stage and response to therapy [33,34], there is a clear potential for this system to be applied in measuring how a patient’s cancer treatment is progressing.
Figure 3. Successful amplification of KRAS G13D mutants further confirmed by a large percentage signal ratio between mutant probe and G13D mutant amplicons.

Figure 4. ctDNA response at different concentrations using DPV peak currents.

The findings of this study on ctDNA amplification are in agreement with several previous studies that were also able to successfully detect ctDNA KRAS mutations in patient samples using ddPCR technique [34–37]. Electrochemical detection will quickly and accurately screen for cancer so treatment can be initiated as quickly as possible. In addition, this study shows the electrochemical sensor can be directly coupled to a standard PCR reaction which employs standard primers and reagents and does not require optimisation, meaning that amplification reactions for other ctDNA markers can be developed off-chip and transferred directly into the assay to produce a ctDNA panel.
The current time to result for cancer detection in a clinical setting is 2–3 days (including sample transportation) for a non-complicated biopsy analysis and 7–10 days for a complicated biopsy analysis [38]. In the UK, National Health Service mutation typing following biopsy can take up to 9 weeks [39]. In total, the DNA isolation from blood, clean up and PCR amplification took approximately 150 min. The ctDNA target incubation takes approximately 60 min while the CV and EIS pre and post hybridisation measurements for each electrode take less than 10 min. This gives a total of 3.5 h. However, through optimisation and device integration, we believe there is making considerable room for optimisation in terms of time to result. The current analysis time of 3.5 h is a big stride towards PoC provision for ctDNA profiling in the healthcare setting. This can be further optimised by isothermal amplification which can cut down the number of thermal cycles and in turn reduce the overall amplification time from 2 h to 1 h [40]. Near future work will explore the detection of other KRAS mutations and mutations in other genes involved in cancer e.g., P53 and BRCA1. Analyzing multiple mutations simultaneously in a given sample without prior knowledge of the alterations using multiplex techniques and direct detection of ctDNA from cancer patient samples will support the future direction of PoC clinical testing.

5. Conclusions

A simple DNA sensor requiring no labelling processes or external indicators was developed using a multi carbon electrode. We were able to design a PCR reaction capable of amplifying either mutant KRAS G13D or wild type KRAS through primer choice from representative patient samples. In parallel, an electrochemical detection scheme involving DNA hybridisation technique and screen-printed carbon electrodes were developed and shown through a series of comparative measurements to be sensitive and specific for the KRAS G13D mutation. Differential pulse voltammetry measurements provided the desired response and showed detection was possible from samples containing as few as 0.58 ng/µL amplicons. In addition, the response was found to be consistent with previously observed results, i.e., large signal decreases being evident upon amplification of the mutant allele, offering the promise of quantitation of mutant sequences from clinical samples. These results raise the prospect of simple, rapid, cost-effective measurement of nucleic acid tumour markers from blood and other body fluids. The current time to result of the electrochemical sensor lies at 4.5 h providing significant scope for optimisation. It is important to note that the sensor being developed can be potentially used for both early detection of cancer and monitoring the response to cancer treatment and thus increases the possibility of tailoring therapy and early diagnosis.

Author Contributions: D.K.C. conceived and supervised the study; B.A. and D.K.C. designed the experiments; B.A. performed the experiments; F.T. and C.P. provided all the clinical information and representative samples; B.A. analysed all the data; B.A. and D.K.C. wrote the paper; and all authors reviewed the results and revised the paper. All authors have read and agreed to the published version of the manuscript.

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References


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