Naked-eye and electrochemical detection of isothermally amplified HOTAIR long non-coding RNA
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An inexpensive, simple and rapid sensor platform capable of detecting cancer-related long non-coding RNA (lncRNA) with high accuracy is of great interest in the field of molecular diagnostics. Herein, we report on the development of a new colorimetric and electrochemical assay platform for long non-coding HOX transcript antisense intergenic RNA (HOTAIR) detection. Isothermal reverse transcription-recombinase polymerase amplification (RT-RPA) was performed to amplify HOTAIR sequences from a RNA pool extracted from a designated number of ovarian cancer cells and a small cohort of plasma samples derived from patients with ovarian cancer. During RT-RPA, biotinylated dUTPs were randomly incorporated in the amplified product. Subsequently, HOTAIR amplicons were magnetically purified and isolated followed by a horseradish peroxidase (HRP)-catalyzed colorimetric reaction in the presence of the 3,3′,5,5′-tetramethylbenzidine (TMB)/H₂O₂ system. We finally introduced three potential readout methods for HOTAIR detection – (i) naked-eye visualisation of the color change for a quick screening of the target, (ii) quantitative absorbance measurement by UV-vis, and (iii) amperometric quantification using the electrochemical properties of TMB. The assay has shown excellent reproducibility (% RSD = <5%, for n = 3) and sensitivity (10 cells/ per mL) while detecting HOTAIR in cancer cell lines and patient samples. The expression of HOTAIR in clinical samples was also verified with a standard RT-qPCR method. We believe that our proof of concept assay may find potential relevance for the routine clinical screening of cancer-associated lncRNAs.

Introduction

Advances in genomic sequencing technologies have revealed the fascinating complexity of transcriptomes. A vast majority of the eukaryotic transcriptome was found to be comprised of non-coding RNAs (ncRNAs), which play a crucial role in the regulation of gene expression.¹ Taxonomically, ncRNAs may be categorised as housekeeping (e.g., ribosomal RNA) and regulatory RNAs (e.g., microRNA and long ncRNA).² Long ncRNAs (lncRNAs), arbitrarily defined as ~200 nt or longer transcripts, represent a subgroup of regulatory ncRNAs involved in chromatin remodelling, epigenomic modulation, as well as the regulation of post-transcriptional gene expression and are increasingly being recognised as pivotal regulators of physiology and pathology.³,⁴ An increasing number of studies have highlighted the significance of lncRNAs as diagnostic and prognostic biomarkers for different types of cancers.⁵-⁷ HOX transcript antisense intergenic RNA (HOTAIR), a 2158 nucleotide long transcript, is among the very few well-characterised lncRNAs which have been reported to be aberrantly expressed in multiple cancers.⁸ HOTAIR influences the chromatin dynamics by interacting with histone modifiers thereby influencing gene expression. Specifically, it binds with the Polycomb Repressive Complex 2 (PRC2) and histone demethylase complex [LSD1 (lysine-specific demethylase 1) and directs their epigenetic modification and gene silencing. In addition to this scaffold function, HOTAIR plays a regulatory role in the
maintenance of protein levels via the ubiquitin-proteasome pathway.9–11 Through these activities, aberrantly expressed HOTAIR may dysregulate multiple genes involved in the pathogenesis of cancer, and thereby promote the initiation, growth and invasiveness of tumors.10 Recent evidence suggests that up-regulated HOTAIR is associated with the proliferation and invasion of tumor cells in breast, liver, ovarian, colorectal, and pancreatic cancers. Moreover, high expression of HOTAIR has been found to be correlated with the survival and prognosis of cancer patients.12–17 Consequently, HOTAIR has become an emerging class of diagnostic or prognostic biomarkers for several types of cancers.

A functional, specific and sensitive detection platform that can readily detect HOTAIR in clinical samples may thus open a principal avenue in diagnostics, prognostics and overall cancer care. Over the past few years, several molecular biology-based approaches such as northern blotting, microarrays, quantitative reverse transcription PCR (RT-qPCR), and next generation RNA-seq have been used for the analysis of HOTAIR and other IncRNAs.18,19 Despite being a specific and commonly used method, northern blotting is susceptible to RNA degradation and requires a relatively large amount of sample input. Moreover, the use of radioactive probes and excess formaldehyde in some of the conventional northern blotting approaches limits their applicability. Although RT-qPCR is considered as more sensitive and reliable, it relies on extensive and expensive instrumentation, and is often affected by the amplification-bias and longer analysis time.1

In this regard, a relatively rapid, sensitive, and inexpensive biosensor-based HOTAIR detection assay could alleviate some of the above-mentioned issues and may represent an appealing alternative for the routine analysis of IncRNA. Electrochemical biosensors have shown great promise in diagnostic applications due to their relatively high sensitivity and specificity, cost-effectiveness and compatibility with the miniaturization.1,20,21 Optical assays comprise another group of highly potential biosensor platforms that are suitable for rapid, direct and label-free analysis of RNA.1 Among optical strategies, the colorimetric method is highly amenable to patient-centric diagnostics in resource-limited settings, where naked-eye evaluation could be useful for the first-pass screening of the analyte. One of the widely used colorimetric systems is the horseradish peroxidase (HRP)/H2O2 coupled with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate. This assay generates a coloured byproduct to signal the presence of the target, given that the target biomolecules are attached with HRP.22 Over the past several years, it was shown that HRP and other enzyme-immobilized nanoparticles (e.g., catalase-coated metal–organic frameworks) have the ability to enhance the performance of bioassays.22–25 Another important element of these colorimetric assays is their chromogenic substrates (e.g., TMB), which often possess the electrochemical properties; hence, it can be further interrogated via an electrochemical quantification approach. Recently few combined colorimetric and electrochemical assay platforms have been demonstrated for RNA detection.26–28

Despite these developments, not many examples of IncRNA biosensing have been reported to date.1,29–31 In addition, to the best of our knowledge, there has been no report on the development of biosensors for the analysis of HOTAIR. One of the major reasons that complicates the biosensing of IncRNAs is their structural instability on the sensor interface (i.e., strong folding tendency of IncRNAs into various secondary or tertiary structures).1 Therefore, biosensing of IncRNA with a conventional RNA-hybridization-based system remains a critical challenge. The present study avoids conventional RNA sensor designs, and reports on the development of a simple colorimetric and electrochemical assay platform for HOTAIR detection. We employed a simple and rapid recombinase polymerase amplification (RPA) isothermal amplification technique to produce biotinylated HOTAIR products. The target amplicons were further magnetically purified using streptavidin-coated HRP and dynabeads, and their level was evaluated with the naked-eye and UV-vis via horseradish peroxidase (HRP)-catalyzed colorimetric readout in the presence of the TMB/H2O2 system. We further used the electroactive properties of TMB to develop an alternative electrochemical assay for more rigorous quantification of HOTAIR. The applicability of our assay has been successfully tested in ovarian cancer cell lines and a small cohort of plasma samples derived from patients with ovarian cancer. The analytical performance of our assay was also found to be in good agreement with the RT-qPCR assay.

**Experimental**

**Reagents and instrumentation**

All the synthetic oligonucleotides were purchased from Integrated DNA Technologies (Singapore) (Table 1). 1-Step TMB substrate solution, HRP-conjugated streptavidin, biotin-11-dUTP solution and Dynabeads MyOne Streptavidin C1 were purchased from Thermo Fisher Scientific (Australia). Reagent grade Tween20, triton-X, and phosphate buffered saline (PBS) tablet (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride) were purchased from Sigma-Aldrich (USA). Ultrapure™ DNase/RNase-free distilled water (Invitrogen, Australia) was used for preparing aqueous solutions. The TwistAmp basic RT-RPA kit (Twist-DX, UK) with pre-included reverse transcriptase was used for isothermal amplification.

A Countess II Automated Cell Counter (Thermo Fisher Scientific, Australia) was used for counting the cells. A

**Table 1** Oligonucleotide sequences used in experiments

<table>
<thead>
<tr>
<th>Oligos</th>
<th>5’-Sequences-3’</th>
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<tbody>
<tr>
<td>HOTAIR Fwd primer sequence</td>
<td>GGTAGAAAAAGCAACCACGAAGC</td>
</tr>
<tr>
<td>HOTAIR Rev primer sequence</td>
<td>ACATAAACCTCTGTCTGTGAGTGCC</td>
</tr>
<tr>
<td>GAPDH Fwd primer sequence</td>
<td>CCGGGAAACTGTGGCGTGATGG</td>
</tr>
<tr>
<td>GAPDH Rev primer sequence</td>
<td>ACATAAACCTCTGTCTGTGAGTGCC</td>
</tr>
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<td>GAPDH Rev primer sequence</td>
<td>ACATAAACCTCTGTCTGTGAGTGCC</td>
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Preparation of RNA from cell line and ovarian cancer samples

SKOV3 ovarian cancer and MeT-5A non-cancerous cell lines were cultured in RPMI-1640 growth medium (Life Technologies, Australia) supplemented with 10% foetal bovine serum (Life Technologies, Australia) and 1% penicillin/streptomycin (Life Technologies, Australia) in a humidified incubator containing 5% CO₂ supply at 37 °C. The SKOV3 and MeT-5A cells were collected after 4 and 7 days, respectively, for subsequent cell counting and RNA extraction.

Staged samples (cross-sectional) were collected at the Ochsner Baptist Medical Center in the clinical trials and obtained via The UQ Centre for Clinical research (UQ IRB 2016000300). Plasma samples were obtained in accordance with the declaration of Helsinki and approved by the Ethics Committee of the University of Queensland and the Ochsner Medical Center (New Orleans, USA). Plasma was separated from whole blood by centrifugation (2000 × 10 min at room temperature) and stored at −80 °C until analyses. Ovarian cancer samples were collected prospectively, assigned according to the histotype classification (e.g. stage I and stage III), and stored at −80 °C in the Biobank units. Only patients with epithelial ovarian cancer high-grade serous subtype (n = 3) and benign samples (n = 2) were included in this study (Table S1†).

RNA was extracted using the miRNeasy Mini Kit (Qiagen, Australia) and TRizol LS Reagent (Life Technologies, Australia) as the lysis solution. A spectrophotometer (SPECTROstar Nano Microplate Reader, BMG Labtech) was used to quantify the RNA concentration. Following a cleanliness check and blank measurement using RNase-free water, 2.0 μL of sample was pipetted onto each microwell plate. The RNA concentration was measured using MARS data analysis microplate reader software.

RT-RPA and colorimetric assay

Synthetic primer sequences for amplifying HOTAIR (for RT-RPA) and GAPDH (housekeeping gene) were designed (Table 1). RT-RPA was performed using the TwistAmp Basic RPA kit (Twist-DX, UK) according to the manufacturer’s instructions with slight modifications. In brief, unless otherwise stated, the RT-RPA master mix contained 29.5 μL of rehydration buffer in one sachet of the kit, 4.8 μL of each primer (500 nM), 4.0 μL of 40 nM biotinylated dUTPs and 11.7 μL of RNase free water to make a 50 μL reaction volume. The volume was aliquoted in 4 tubes and 1.0 μL of template RNA (15 ng) and 0.625 μL of 280 mM MgAc were added before incubating at 43 °C for 20 min. The amplified products were immediately stored at −20 °C for further replicate analysis and gel electrophoresis.

2.0 μL of RT-RPA products was taken and mixed with 1.0 μL of 1: 2000 diluted streptavidin (SA)-HRP, 1.0 μL of SA-magnetic beads and 10 μL of wash buffer-1 (0.5% Triton-X in 10 mM PBS) for 10 min. A magnetic rack was subsequently used to separate the beads. The beads were washed twice with wash buffer-2 (0.1% Tween20 in 10 mM PBS). After magnetic purification of the beads, 35 μL of 1-step TMB substrate solution was added to the beads and incubated for 10 min to observe the color change. Next, using an external magnet, the clear blue solution was separated, and absorbance (at 652 nm) data were obtained with a spectrophotometer. Please note that we have demonstrated the current proof of concept electrochemical and colorimetric assays using RT-RPA HOTAIR amplicons only. The methods however can be extended towards the biosensing of the housekeeping gene (e.g., GAPDH) for a relative quantification of HOTAIR.

Electrochemical readout

CV was performed in 10 mM PBS solution containing 2.0 mM [K₂Fe(CN)₆] electrolyte solution for determining the effective surface area of the electrode as shown before (see ESI†). For the electrochemical detection of RT-RPA products, 1.0 μL of stop solution was added to the blue-colored solution described above, which turned yellow upon the cessation of the reaction. Then, 35 μL of the resulting mixture was pipetted onto the SPGE surface for amperometry measurements at 150 mV for 80 s.

Results and discussion

The assay protocol for the isolation and detection of HOTAIR is schematically presented in Fig. 1. In our assay, isothermal RT-RPA amplified HOTAIR was detected using TMB-based colorimetric and electrochemical readouts. Briefly, total RNA was extracted from cell lines and plasma samples derived from patients with ovarian cancer. During RT-RPA, biotinylated dUTP bases were randomly inserted into the amplified strands. Following RT-RPA, SA-coated Dynabeads and SA-HRP were dispersed into the amplified product, which selectively bind to the biotinylated strands of HOTAIR due to the well-established high-affinity interaction between biotin and SA. The target RNA was then isolated and purified with multiple magnetic washing steps via the Dynabead-based magnetic separation protocol. Upon the addition of the 1-step TMB solution, the HRP present in the isolated HOTAIR-Dynabeads-HRP conjugates triggers the oxidation of TMB that generates a blue-colored charge transfer complex. This facilitated the naked-eye observation of the presence of HOTAIR. The intensity of the colored complex is likely to be proportional to the
amount of captured HRP present in the conjugates, which is in turn proportional to the amount of HOTAIR in the amplified RNA sample. The color intensity was also quantified by UV–vis at 652 nm. With further addition of a stop solution (acid), the blue colored product converted to a stable electroactive yellow (diimine) complex, which enabled an alternative amperometric quantification of HOTAIR.

The attachment of biomaterials with biotinylated HOTAIR amplicons was investigated by AFM (Fig. S1†). The image shows distinct differences between the control (no biotinylated amplicon, Fig. S1A†) and sample (biotinylated HOTAIR amplicons/SA-Dynabeads/SA-HRP). To demonstrate the assay specificity, we performed our assay with RNA extracted from the same number (10 000 cells) of two different cell lines. SKOV3 is an ovarian cancer cell line where HOTAIR has been reported to be overexpressed, while MeT-5A is a non-cancerous cell line. Apart from the no-template (NoT) control, we performed an additional control experiment where we did not add biotinylated d-UTP during the RT-RPA (No b-dUTP). As can be seen in Fig. 2A, the control experiment without the target (NoT) produces no color and a very low response in the UV-vis measurement (absorbance at 652 = 0.034). The second control experiment, where we did not include b-dUTP, also did not generate any noticeable color change, and the UV-vis measurement showed a similar level of low response (absorbance at 652 = 0.075). These control studies clearly demonstrate that our method completely relies on the presence of biotinylated HOTAIR in the amplified products, followed by their magnetic separation via the SA–biotin interaction. The data further confirm that our magnetic separation protocol and the overall

Fig. 1 Schematic of the colorimetric and electrochemical detection of HOTAIR assay. Isothermal RT-RPA was performed on extracted total RNA. During amplification, biotinylated dUTPs were inserted into the amplicons. Using SA-Dynabeads and SA-HRP, the biotinylated HOTAIR was magnetically purified and separated. Subsequently, the employment of the HRP/TMB-based colorimetric reaction facilitated the naked-eye and UV-vis readout while the electrochemical properties of TMB enabled a more precise electrochemical quantification of HOTAIR.

Fig. 2 Specificity of the colorimetric and electrochemical assay. (A) Absorbance (UV-vis) obtained for SKOV3 (ovarian cancer) and MeT-5A (non-cancerous) cell lines, no biotinylated d-UTP bases (No b-dUTP) and no-template (NoT) controls (inset: pictures of the naked-eye detection); (B) representative i–t curves of amperometric current density for HOTAIR detection. Error bars represent the standard deviation of three independent experiments.
assay were not susceptible to non-specific interaction from other types of non-specific RNAs present in the bulk RNA samples, thereby attributing high specificity to the assay.

When the assay was run with SKOV3 cancer cell lines, it consistently produced an absorbance which was ~50 times higher than that of the NoT (absorbance at 652 = 1.809 vs. 0.035). However, in the assay with the non-cancerous Met-5A cell line, a 5-times lower response in the UV-vis data was recorded (absorbance at 652 = 1.809 vs. 0.352). Also as shown in the picture (Fig. 2A inset), it is evident that all the control and cell line samples can be visually discerned with the naked eye. When we performed the electrochemical experiment, we found a similar trend of the amperometric response (Fig. 2B), where the current response obtained with SKOV3 is several fold higher than that of No-dUTP and NoT controls (2.2 vs. 0.22 and 0.1 μA cm⁻²). Altogether, these control experiments clearly demonstrate the excellent level of specificity of our assay towards the detection of HOTAIR. Furthermore, we performed gel electrophoresis of the RT-RPA products to verify the HOTAIR primer specificity. As can be seen in Fig. S2,† from the same starting amount of RT-RPA products, clear bands for specific 170 bp products were observed for both SKOV3 and Met-5A samples, while the control experiments without the template (NoT) did not generate any RPA products.

To evaluate the sensitivity of our assay, total RNAs isolated from a known number of SKOV3 cell line samples obtained via serial dilution (10:1, 100:1, 1000:1 and 10 000:1 cells per mL) were tested. From the picture of Fig. 3A (left panel), a gradual increment of the color intensity with increasing cell numbers could be visually observed. Moreover, with the naked eye, the color corresponding to the RNA sample of 10 cells (light blue) can easily be visually discriminated from that of the NoT (no color change). Afterwards, when the subtle color changes of the assay were quantified via UV-vis readout, a similar increasing trend of the absorbance value was observed in the RNA sample collected across the range of 0–10 000 SKOV3 cells (Fig. 3A, right panel). This linear increase of the colorimetric response is due to the presence of a higher amount of RT-RPA biotinylated HOTAIR products with the increasing number of cancer cells. These amplicons in turn can attract a large number of streptavidin-HRP, which subsequently accelerate the rate of TMB oxidation and thus can increase the intensity of the blue-colored complex. As can be seen in Fig. 3A, the linear regression equation for the colorimetric assay was estimated to be $y = 0.0468x - 0.1006$, with the correlation coefficient ($r^2$) of 0.9602. The data also show that our assay can successfully detect HOTAIR from RNA samples extracted from a very low number of cells (10 cells per mL), with a very high signal to noise ratio of ~4.5 (absorbance at 652 nm = 0.034 vs. 0.156).

In the case of electrochemical readout, similar to the UV-vis data, a gradual increase in the current density profile with an
increasing amount of cells was recorded (Fig. 3B). The linear regression equation for amperometric readout was estimated as \( y = 0.49x + 0.176 \), with an \( r^2 \) of 0.9942. We also found that this readout could detect HOTAIR from RNA samples extracted from as low as 10 cells per mL, however with a relatively better signal to noise ratio of 6.8. It is noteworthy that this low level of LOD is adequate for the clinical screening of HOTAIR.

To the best of our knowledge, until now, there are no previous reports on the colorimetric and electrochemical detection of HOTAIR. However, there are only a few reports on the electrochemical detection of other lncRNAs. Among these, assays developed by Pu and colleagues can be considered as some of the most prominent and have been reported to detect ‘highly up-regulated in liver cancer’ (HULC) and ‘nuclear paraspeckle assembly transcript 1’ (NEAT1) long non coding RNA.\(^2^9,30\) In these reports, to enhance the assay performance, electrochemical sensors were functionalised with especially designed nanostructured materials and components (e.g., green l-cysteine electrodeposition, tagging with Au–Rh hollow nanospheres, designing PtPd nanodendrite/nano-flowerlike graphene oxide, etc.). Our assay avoids such time-consuming and multi-step sensor fabrication procedures and rather adopted a much simpler naked-eye colorimetric assessment followed by an electrochemical readout. By employing unmodified and easy-to-use disposable electrodes, our report offers a sensitive and selective platform for lncRNA detection.

To demonstrate the applicability of our assay in complex biological samples, we further performed our assay on five plasma samples derived from patients with ovarian cancer. Only patients with epithelial ovarian cancer high-grade serous subtype \((n = 3)\) and benign samples \((n = 2)\) were tested (Table S1†). As shown in Fig. 4A, compared to the NoT control, absorbance data corresponding to the high grade epithelial ovarian cancer samples \((P1, P2, P3)\) produced at least 75-times higher response (absorbance at 652 nm = 0.034 vs. 2.882/2.702/2.519). It is also noticeable that the absorbance obtained from P1, P2, P3 was at least two times higher than that of the benign samples \((P4 \text{ and } P5)\). The current density profile obtained for the electrochemical readout (Fig. 4B) also followed a similar trend of colorimetric data. The data derived from these clinical samples also showed an excellent inter-assay reproducibility \((\text{RSD} \leq 5\%\), for \(n = 3\)) for the detection of HOTAIR, which is better than or comparable to most of the existing RNA sensors.\(^1,2^5,3^4,3^5,3^7,3^8\) We also verified HOTAIR expression in clinical samples via RT-qPCR (see the ESI†).

**Conclusions**

We have developed a simple naked-eye colorimetric and electrochemical approach based on an innovative merging of isothermal RT-RPA and HRP-catalyzed colorimetric and electrochemical readouts. Isothermally amplified and magnetically purified and isolated HOTAIR sequences were detected by HRP-catalyzed colorimetric reaction in the presence of the TMB/H\(_2\)O\(_2\) system. The initial readout was obtained with a visual observation of the color change of the assay which allows a quick first pass screening (yes/no answer) of HOTAIR. This was followed by a quantitative absorbance measurement by UV-vis (at 652 nm). The versatility of the developed assay platform was further demonstrated with an amperometric readout which quantified HOTAIR with more precision. The data obtained with our proof of concept assay which integrates these three useful readout platforms were found to be in good accordance with a standard RT-qPCR, while the assay also showed excellent reproducibility \((\% \text{ RSD} = <5\%\), for \(n = 3\)) and sensitivity \((10 \text{ cells/ per mL})\) in cancer cell lines and patient samples.

Overall, there are some distinct advantages in our assays. First, the assay was able to detect HOTAIR in the human
plasma samples, which demonstrates its high potentiality towards liquid biopsy of cancer. Second, the RT-RPA isothermal assay allows us to avoid an expensive and time-consuming PCR amplification setup, and enable the rapid amplification of HOTAIR within 20 minutes at a manageable temperature in the presence of minimal resources. Third, multiple magnetic mixing and purification steps to isolate the HOTAIR amplicons may reduce the matrix effects of the biological samples contributing to the diminution of non-specific detection. Fourth, the assay eliminates the need for tedious cleaning procedures of traditional disk electrodes as it uses the inexpensive disposable SPGE. Finally, considering the minimal resource requirement of the colorimetric sensor, along with the versatility and sensitivity of the electrochemical method, we envision that our assay would find potential clinical applications for sensitive and specific analysis of IncRNA for screening human diseases.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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Notes and references

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