ANRIL as a prognostic biomarker in colon pre-cancerous lesion detection via non-invasive sampling

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Long non-coding RNAs have been proposed as biomarkers for the detection, prevention and screening of various malignancies. In this study, two lncRNAs (ANRIL and BANCR) were assessed for biomarker application in the early detection of colorectal cancer (CRC) through stool specimen testing, as a non-invasive and cost-effective methodology. A total of 40 stool samples were collected from patients referred to the hospital with colorectal cancer or adenomatous polyps as pre-cancerous lesions; patients were diagnosed using colonoscopy and pathology reports were available. Twenty control samples were also obtained from healthy subjects for comparison. RNA extraction and cDNA synthesis were followed by real-time PCR to evaluate lncRNA expression. The up-regulation of ANRIL in 20% of samples taken from polyp patients, combined with up-regulation in 65% of patients with CRC, confirmed the potential usefulness of ANRIL as a prognostic biomarker (AUC 0.95; P < 0.0001). BANCR relative expression analysis illustrated significant up-regulation in polyp (P < 0.04) and tumoural participants (P < 0.03) compared with normal control individuals. The expression patterns of ANRIL and BANCR in polyp cases were significantly correlated according to correlation analysis (r = 0.45, P < 0.045). ANRIL expression patterns in stool specimens of polyp and tumour cases supported the use of ANRIL as a prognostic biomarker for screening patients in the early stages of CRC. Up-regulation of BANCR in pre-cancerous lesions as well as down-regulation of ANRIL may also be a specific marker pair for easy, convenient and fast CRC prognosis.

Key words: ANRIL, BANCR, colorectal cancer, long non-coding RNA

INTRODUCTION

In 2020, approximately 1.93 million individuals globally were diagnosed with colorectal cancer, and nearly one million were estimated to have died from this cancer (Xi and Xu, 2021). In a recent study, CRC was rated as the third highest cancer type in men and women in the USA, accounting for around 9% of all cancers, and 17,930 cases and 3,640 deaths were anticipated in 2020 among individuals aged less than 50 years (Siegel et al., 2020). Although developing countries are classed as low-risk for CRC compared to developed countries, the rate of onset has been increasing in recent decades. More recently, the occurrence of this malignancy has been rising among younger people, highlighting CRC as a major public health burden (Ahmadi Lari, 2020).
Despite the chance of recovery for patients who are in the early stages of CRC being more than 90% (Sun et al., 2016a), unfortunately, colorectal cancer is most commonly detected in more advanced stages (Karthik et al., 2014). Therefore, early detection of malignancy is important, and the use of non-invasive methods is preferable (Das et al., 2017; Rejali et al., 2021).

Long non-coding RNAs (lncRNAs) have been found to play a crucial role in diverse biological processes through interactions with other cellular molecules, including DNA, RNA and proteins, via multiple pathways (de Bony et al., 2018; Ming et al., 2021). Dysregulation of lncRNAs has been reported in a variety of cancers including CRC (Fang and Fullwood, 2016; Yang et al., 2017; Cao et al., 2021; Melixetian et al., 2021; Wang et al., 2021). Since genetic/epigenetic and environmental factors are involved in the development and progression of CRC (Toiyama et al., 2014), lncRNAs have become targets of interest for diagnostic, prognostic and therapeutic applications (Luo et al., 2017). Recent studies on lncRNAs have also revealed that they can act as tumour suppressors or oncogenes, and gene expression can become activated or be inhibited by lncRNA functions (Xie et al., 2016). Therefore, defining lncRNA function in tumourgenesis is now a priority.

Clinical diagnostic biomarkers such as CEA and CA199 have been proposed previously for CRC, but have not demonstrated sufficient sensitivity or specificity for early detection in CRC (Zou et al., 2017). The evaluation of efficient biomarkers to improve screening and early detection in CRC is therefore now a priority (Nissan et al., 2012; Yang et al., 2017).

The lncRNA ANRIL (antisense non-coding RNA in the INK4 locus) is located on chromosome 9 in humans (9p21.3) and consists of 21 exons within the CDKN2B–CDKN2A gene cluster. CDKN2B and CDKN2A have well-established roles in cell proliferation, apoptosis, senescence and aging (Pasmant et al., 2007). This region is a notable genetic susceptibility locus for several cancers (Green et al., 1996). ANRIL plays a crucial role in gene regulation and histone modification, and is thought to participate in the tumour microenvironment through its involvement in extracellular matrix remodelling, thereby aiding metastasis (Mehta-Mujoo et al., 2019).

Along with ANRIL, the other lncRNA evaluated in the present study is BRAF-activated non-coding RNA (BANCR), a lncRNA with four exons located on chromosome 9 (9q21). BANCR is closely associated with V600EBRAF, the most frequent mutation type of the BRAF gene, which has also been detected in approximately 5–22% of CRC cases (Brose et al., 2002; Yang et al., 2014). BANCR is frequently deregulated in various human cancers (Yu et al., 2017). In colorectal cancer studies, contradictory results indicate that BANCR can act as an oncogene or a tumour suppressor gene, based on contrasting evidence (Brose et al., 2002; Liao et al., 2017).

lncRNAs have previously been sampled and investigated in human body fluids such as blood, ejaculate and urine (Zhang et al., 2013). However, this is the first study to examine lncRNA expression in CRC stool samples.

In this study, we evaluated the expression level of two lncRNAs, ANRIL and BANCR, in stool specimens. Stool samples provide a very good indication of the colon area, with the possibility of CRC tumoural cell presence (Davies et al., 2005). Furthermore, there are certain advantages for stool analysis: it is a non-invasive sampling technique; there is no need for bowel preparation; it enables screening of the entire length of the colon and rectum; and it produces specimens that are easily transportable (De Maio et al., 2014). Taking these considerations together, the discovery of molecular biomarkers in stool specimens could offer beneficial new options in providing early continuous surveillance of CRC.

**RESULTS**

**Evaluating the expression of BANCR and ANRIL in tumour and polyp faecal samples** qRT-PCR was performed to measure BANCR mRNA expression levels in 20 stool samples collected from CRC patients, 20 from individuals diagnosed with adenomatous polyps and 20 from healthy normal faecal samples. Patients in each disease category were divided into two groups of up-regulated and down-regulated expression of lncRNAs (BANCR, ANRIL) by calculating relative quantification (RQ) and mean of RQ. The expression level of the lncRNA BANCR was significantly up-regulated in polyp samples ($P < 0.0003$; Table 1). Besides, the correlations between BANCR expression in tumour or polyp samples and the patients’ clinicopathological parameters (including sex, age, location, history of colon disease, family history of CRC, diabetes, smoking and alcohol consumption) were measured to determine their clinical significance.

| Disease Category | BANCR | ANRIL |
|------------------|-------|-------|
|                   | LOW n (%) | HIGH n (%) | $P$ value | LOW n (%) | HIGH n (%) | $P$ value |
| Polyp stool specimen | 6 (30%) | 14 (70%) | 0.0003* | 16 (80%) | 4 (20%) | < 0.0001* |
| Tumour stool specimen | 13 (65%) | 7 (35%) | < 0.0001* | 7 (35%) | 13 (65%) | < 0.0001* |

*, Statistically significant ($P < 0.05$).
Table 2. ANRIL and BANCR association with clinicopathological characteristics in stool samples of polyp and tumour participants

| Characteristics          | Tumour   |          |          | Polyp     |          |
|--------------------------|----------|----------|----------|-----------|----------|
|                          | BANCR    | ANRIL    |          | BANCR     | ANRIL    |
|                          | Mean ± SD of RQ | Mean ± SD of RQ | P value | Mean ± SD of RQ | Mean ± SD of RQ | P value |
|                          |          |          |          |          |          |
| **Sex**                  |          |          |          |          |          |
| Male                     | 2.32±1.47 | 1.89±2.30 | 0.90     | 0.59±0.49 | 1.68±1.17 | 0.19     |
| Female                   | 1.17±1.22 | 2.32±3.46 | 0.16     | 0.36±0.35 | 0.05±0.04 |          |
| **Age**                  |          |          |          |          |          |
| < 50                     | 0.50±0.96 | 4.11±2.69 | 0.03a    | 0.28±0.41 | 0.20±0.45 | 0.47     |
| > 50                     | 1.57±2.00 | 3.08±2.10 | 0.38     | 0.49±0.51 | 0.73±1.46 |          |
| **Location**             |          |          |          |          |          |
| Cecum                    | 0.51±0.49 | 3.11±2.87 |          | 0.45±0.43 | 0.22±0.25 |          |
| Ascending                | 0.07±0.05 | 4.70±1.55 | 0.23     | 0.43±0.46 | 0.04±0.0  |          |
| Sigmoid                  | 1.02±1.32 | 7.20±0.00 | 0.04     | 0.22±0.34 | 0.5±0.67  | 0.48     |
| Descending               | 0.24±0.40 | 2.20±2.49 | 0.16     | 0.33±0.33 | 0.74±1.82 |          |
| Rectal                   | 2.36±2.46 | 3.03±1.95 | 0.05     | 0.45±0.42 | 0.03±0.0  |          |
| **History of colon disease** |          |          | 0.05     | 0.01*    |          | 0.35     |
| Yes                      | 3.80±2.93 | 1.12±1.88 |          | 0.03±0.04 | 1.21±2.01 |          |
| No                       | 0.82±0.91 | 3.82±2.04 | 0.21     | 0.61±0.54 | 0.42±0.90 |          |
| **Family history of CRC** |          |          |          |          |          |
| Yes                      | 1.56±2.96 | 2.80±1.98 | 0.21     | 0.22±0.16 | 0.002±0.004 | 0.04*   |
| No                       | 1.49±1.67 | 3.36±2.31 | 0.21     | 0.53±0.51 | 0.67±1.35 |          |
| **Diabetic history**     |          |          |          |          |          |
| Yes                      | 3.70±2.41 | 0.03±0.01 | 0.35     | 0.07±0.10 | 0.02±0.03 | 0.16     |
| No                       | 1.09±1.58 | 3.70±2.09 | 0.01a    | 0.47±0.49 | 0.60±1.29 |          |
| **Smoking**              |          |          |          |          |          |
| Yes                      | 2.2±13.14 | 2.36±2.30 | 0.09     | 0.09±0.06 | 0.13±0.23 | 0.26     |
| No                       | 1.18±1.04 | 3.63±2.09 | 0.18     | 0.55±0.50 | 0.65±1.36 |          |
| **Alcohol consumption**  |          |          |          |          |          |
| Yes                      | 0.40±0.08 | 4.30±1.41 | 0.49     | 0.38±0.40 | 1.08±1.28 | 0.09     |
| No                       | 1.63±2.00 | 3.04±2.26 | 0.70     | 0.45±0.52 | 0.37±1.25 |          |

* Statistically significant (P < 0.05).

Table 1. Regulation of ANRIL in tumour patients' faecal samples

| Characteristics | Tumour Polyp | Tumour Normal | Polyp Anogenital | Polyp Normal |
|-----------------|--------------|---------------|-----------------|-------------|
| Fasting glucose| 8.2±2.0      | 6.8±1.5       | 7.9±1.8         | 6.5±1.3     |
| 2-h post-glucose| 10.6±2.5     | 9.2±1.8       | 10.1±2.4        | 9.3±1.7     |
| HbA1c           | 6.5±1.2      | 6.1±1.1       | 6.6±1.3         | 6.2±1.2     |
| BMI             | 25.3±2.1     | 24.0±1.8      | 25.7±2.3        | 24.5±1.9    |

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The expression level of BANCR in polyp and tumoural specimens was significantly correlated with history of colon disease (P < 0.01, P < 0.04), and specifically in tumour samples with diabetic history and age (P < 0.04, P < 0.03). BANCR was up-regulated in 70% (14/20) of the patients with adenomatous polyps and 35% (7/20) of CRC patients (Table 1).

Furthermore, mRNA expression analysis of ANRIL in specimens collected from confirmed polyp diagnosis participants and CRC patients demonstrated the up-regulation of ANRIL in tumour patients' faecal samples (Table 1). This was in contrast to down-regulation in faeces samples from individuals with a family history of CRC in polyp and diabetic history in tumoural cases (Table 2). Although 20% of polyp samples illustrated up-regulation, 80% exhibited down-regulation while 35% were down-regulated via qRT-PCR non-parametric analysis. Relative expression of ANRIL and BANCR in normal, polyp and tumoural samples were compared (Fig. 1). There was a significant difference between normal and polyp samples (P < 0.03, P < 0.04, respectively), and also polyp and tumour specimens (P < 0.02, P < 0.03, respectively), in both ANRIL and BANCR expression levels. A significant difference was also seen between tumour and normal samples in BANCR (P < 0.03).

**AUROC evaluation of BANCR and ANRIL in polyp and tumour stool specimens** For evaluating the characteristics of BANCR and ANRIL as effective biomarkers for polyp detection, area under ROC curves
(AUROC) were prepared for 40 individuals (20 polyp and 20 colon tumour patients). The ANRIL ROC curve demonstrated a strong separation between faecal samples in participants diagnosed with adenomatous polyps and stool samples of CRC patients, with an AUC (area under the curve) of 0.95 (95% confidence interval (CI) 0.88–1.00, $P < 0.0001$). The sensitivity of the test for $RQ < 1.6$ was 95.24% with 80% specificity. BANCR displayed a weaker correlation: the AUC was 0.66 (95% CI 0.50–0.82, $P = 0.056$). Sensitivity of 59% and specificity of 61% were estimated for $RQ < 0.25$ (Fig. 2).

**Correlation evaluation between ANRIL and BANCR in tumour and polyp samples** To elucidate the association between the expression of ANRIL and of BANCR, the relative expression values of the IncRNAs were compared in each sample set. A significant association with positive correlation was observed in polyp specimens ($r = 0.45; P < 0.045$). There was a negative relationship between the relative expression levels of ANRIL and BANCR in tumour faeces samples, but no significant correlation was found ($r = -0.08; P < 0.76$) (Fig. 3).

**DISCUSSION**
Early detection of colorectal cancer (CRC) is a priority goal for screening high-risk patients and increasing long-term survival. The quality of CRC screening is currently insufficient for global utilization, with low sensitivity and specificity of testing via conventional stool-based screening. Furthermore, accelerated expenditure with low participation compliance in colonoscopy points to the need for...
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novel biomarker development. Long non-coding RNAs represent an excellent biomarker candidate in stool specimens; this sampling method is non-invasive and involves no risk of colonoscopy side effects for patients. Conventional stool tests are easy, require no preparation, and can be repeated at short intervals with low cost, and should therefore also result in an elevation in compliance rates.

ncRNAs play key roles in various cellular functions such as proliferation, differentiation, migration, angiogenesis and apoptosis. Recent studies have shown that abnormal expression of ncRNAs is correlated with different cancers, including CRC. The finding that ncRNAs are stable in stool, blood plasma and serum highlights the opportunity for developing novel innovative procedures using ncRNAs as early diagnostic biomarkers in CRC.

lncRNAs can be considerably more sensitive and specific for diagnosis than genomic DNA, mRNA or protein biomarkers (Slaby, 2016). lncRNA function and expression patterns are diverse in cancerous and pre-cancerous lesions. Hence, lncRNA expression evaluation in individuals with confirmed adenomatous polyps and colon cancer is a potentially important biomarker, implementable through non-invasive methodologies for early detection of CRC.

To our knowledge, the data presented here constitute the first investigation to evaluate the lncRNAs BANCR and ANRIL in faecal samples of CRC patients and diagnosed polyp cases. However, similar studies have been performed on colorectal tumour tissue, melanoma and lung cancer to investigate BANCR's function in biological processes including proliferation, migration and invasion (Yang et al., 2014; Jiang et al., 2015). BANCR and ANRIL expression as well as clinicopathological parameters of the analysed samples were evaluated. BANCR was up-regulated in faecal samples taken from both adenomatous polyp patients and CRC patients, but the AUROC plots for detecting an association between lncRNA expression in the two sample groups revealed no significant correlation. BANCR lncRNA up-regulation was associated with lymph node metastasis and poor survival rate among colorectal cancer patients by Shen et al. (2017), but no evidence of a correlation with age was reported. Lou et al. (2018) reported significant overexpression of BANCR in breast tumour tissues relative to para-carcinoma normal tissues. They showed that patients who overexpressed BANCR had a poor prognosis compared with patients having low expression of BANCR. In addition, Zhou and Gao (2016) described a key role for up-regulated BANCR in the occurrence and development of hepatocellular carcinoma and the prognosis of affected patients, proposing its application as an lncRNA biomarker for early diagnosis of cancer and prognosis screening. A recently published meta-analysis reported the association of an undesirable prognosis for most cancer patients with elevated BANCR expression (Fang et al., 2020).

Sun et al. (2016b) showed higher ANRIL expression in CRC tissue compared with adjacent non-tumour tissues. The overexpression was significantly correlated with a reduction in survival rate. They further predicted that ANRIL may be a primary participant in the advancement of CRC, which agrees with our own data presented here evaluating the expression of ANRIL in polyps and tumours of defined samples. The up-regulation of ANRIL lncRNA in polyps, which are pre-cancerous lesions, confirmed the hypothesis of ANRIL playing a role in the early stage of the disease. Furthermore, the significant correlation between ANRIL relative expression in samples from polyp patients and CRC patients verified ANRIL as a candidate prognostic biomarker in

![Fig. 3. Correlation analysis was performed between relative quantification (RQ) of selected lncRNAs in tumour patients’ stool samples and in primary lesion (polyp) patients’ specimens. (A) Association between ANRIL and BANCR yielded r = –0.08 with non-significant P < 0.76 in fecal samples of CRC patients. (B) The RQ relationship of lncRNAs in patients with polyps showed a positive correlation r = 0.45 with significant P < 0.045.](image-url)
CRC. Uniquely in our study, a trend for down-regulation of ANRIL in samples of CRC patients was found, but not observed to be significant. On the other hand, pathology reports of CRC patients noted that all patients with CRC who entered our study were diagnosed in the early stages of disease. Hence, ANRIL expression may be confined to the later stages of CRC; however, expression may also be variable in different sample types. These findings illustrate the complex and disparate roles of ANRIL in CRC.

Using ANRIL lncRNA as a prognostic biomarker for detecting adenomatous polyps from faecal samples will shed light on the early detection of CRC via non-invasive, cost-effective and simple methodology, improving patient outcomes.

CONCLUSIONS

Different diagnostic and prognostic methodologies for the early detection of colorectal cancer are available. Most routine methods are, however, invasive and expensive. Since the deregulation of lncRNAs in cancerous tissues has been observed in faecal samples, these factors are recognized as non-invasive and preferable biomarker candidates, with acceptable tolerance for affected or at-risk patients compared with biopsy administration. Further research is required into the fundamental mechanisms and functions of BANCR and ANRIL lncRNAs in CRC.

MATERIALS AND METHODS

Patients Forty stool samples, including 20 from CRC patients, plus 20 from adenomatous polyp patients referred to the hospital with histological confirmation of the disease, were randomly collected. A further 20 samples from healthy individuals who underwent routine screening examination were included as control samples. Patients with a record of chemotherapy or radiotherapy in their history were omitted from the survey. Informed consent was obtained from all subjects involved in the study, which was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board Institutional Research Ethics Committee of Taleghani Hospital, Tehran (ethical approval number: IR.SBMU.RIGLD.REC.1397.187). Collected samples were kept in EDTA buffer at −80 °C until the time of extraction. The clinical features of patients were verified by an expert pathologist. All relevant information including age, sex, weight, body mass index, smoking and alcohol consumption, blood in faeces, diabetes and a history of colon cancer and chronic inflammatory disease was recorded by questionnaire.

Stool RNA extraction Total RNA was extracted from stool samples using the Qiagen RNeasy Plus Mini Kit according to the manufacturer’s instruction, with attention to usage of DNase/RNase-free devices and solutions. After extraction, the quantity and quality of RNA was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and electrophoresis in a 1% agarose gel. The optical density 260/230 nm and 260/280 nm ratios were determined using a NanoDrop application to ensure RNA purity. RNAs were then reverse transcribed into cDNA using the Reverse Transcription Kit (Yekta Tajhiz).

Quantitative real-time PCR (qRT-PCR) qRT-PCR reactions were carried out using 75 ng of cDNA, 10 μl of 2×SYBR Green (Takara) and 200 nM forward and reverse primers in an ABI 7500 Real-Time PCR instrument (Applied Biosystems). 18S rRNAs were included as internal controls.

PCR conditions were 52 s at 95 °C for initial denaturation, 40 cycles of 28 s at 95 °C for denaturation and 36 s at annealing temperature 56.5 °C. The sequences of primers used in the qRT-PCR reaction for BANCR were 5′-ACAGGACTCTCATGGCAAAAGC-3′ as the forward primer and 5′-ATGAGAAAAAGCTTGGCCAGT-3′ as the reverse primer (Yin et al., 2019). ANRIL forward and reverse primer sequences were 5′-CCGGCTCCCCTTTACCTCCTTA-3′ and 5′-CTGATTGCGGGATAGARGA-A3′, respectively (Chen et al., 2014). Ct values of selected lncRNAs were normalized according to 18S rRNA (forward and reverse primers 5′-GAGAAGGCTACACACTCC-3′ and 5′-GAGAAGGCTACACACTCC-3′, respectively) as previously described (Sakurai et al., 2008), via 2−ΔΔCT method analysis.

Statistical analysis Statistical analyses were carried out using SPSS software version 23 (SPSS) and GraphPad Prism 8.0. The Mann–Whitney test was applied to compare the expression of BANCR and ANRIL in faecal specimens of cancerous patients, and of patients diagnosed with colorectal adenomatous polyps, with that in healthy normal stool samples. Student’s t-test or one-way ANOVA was used to determine the correlation between BANCR or ANRIL expression and clinicopathological variables. The Spearman method was used for reporting (r) in correlation analysis. The resulting data are reported as mean ± standard deviation (SD) of RQ. Statistical significance was defined as P < 0.05.

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