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Research Article

A Long noncoding RNA, ANCR, is Upregulated in Bladder and Breast Tumor Tissues

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Abstract

Long non-coding RNAs (lncRNAs) have recently found to have important regulatory roles, and their aberrant expressions and functions are directly linked to carcinogenesis. Both urinary bladder and breast tumors are prevalent neoplasms, with high rates of incidence. To identify a potential expression alteration of the recently discovered "anti-differentiation non-coding RNA, (ANCR), during tumorigenesis, we initially assessed its expression in several cancer cell lines (LNCAP, MCF-7, Ht-29, 5637, A549, HepG2, and PC3) and then compared its expression variability in tumor vs. non-tumor samples of bladder and breast. Here, ANCR expression profile was studied by qRT-PCR in paired tumor and marginal non-tumor samples obtained from patients that had been referred to the Labbafi-Nejad and Imam Khomeini Hospitals, respectively. Our data revealed a significant upregulation ($p = 0.003$) of ANCR in breast tumor tissues, in comparison to non-tumor marginal specimens from same patients. Similar upregulation was also detected in bladder tumor samples, however, this alteration was not statistically significant ($p \geq 0.05$), probably due to small number of samples (n = 10). In conclusion, our results suggest a possible role of ANCR in tumorigenesis of bladder and breast tissues, as well as its potential usefulness as a novel diagnostic biomarker for bladder and breast tumors.

***Keywords*:** lncRNA, ANCR, Breast Cancer, Bladder Cancer

Introduction

Long non-coding RNAs, > 200 nucleotide- non coding RNAs, are transcribed extensively throughout the genome, and regulate various life processes including development, differentiation, and pluripotency. They regulate these processes mostly by fine tuning gene expression, and cellular mechanisms such as chromatin modification, transcription and posttranscriptional processes (Gabory et al., 2010; Gupta et al., 2010; Mercer et al., 2009; Tripathi et al., 2010). Due to these functions, lncRNAs are emerging as critical players in tumorigenesis and several studies have reported lncRNAs deregulation in a number of cancers (Congrains et al., 2013; Huarte et al., 2010; Huarte and Rinn, 2010; Nakano et al., 2006; Redon et al., 2010). Such studies have demonstrated that this class of the non-coding RNAs contains molecules with both oncogenic and tumor suppressive functions, suggesting that their aberrant expressions may be a contributor in carcinogenesis. Urinary bladder cancer (UBC) is the 9th most prevalent cancer around the world (Murta-Nascimento et al., 2007), with the rate of incidence in males being much higher than in females. Annually about 261000 new cases are diagnosed with UBC, and roughly 115000 patients die from it, mostly due to a late diagnosis (Christoforidou et al., 2013; Larsson et al., 2008). The difference between the incidence and mortality rates suggests that UBC has a long progression period (Murta-Nascimento et al., 2007). UBC is mostly common in developed countries (Murta-Nascimento et al., 2007), while in Iran the rate of incidence is moderate accounting to <8.8/100000 in males and < 2.8/ 100000 in females (Parkin et al., 2001).

Breast cancer is also the most prevalent cancer among women and ranks the 2nd, after lung cancer. However, due to a relatively good prognostic procedure, it ranks fifth in the list of cancer-caused death (Parkin et al., 2001; Sharif et al., 2010). In

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Iran breast cancer is also the most common cancer among women, comprising about 21.4% of all cancers affecting female population (Noroozi et al., 2011). Moreover, it has been shown that in Iran younger women are at relatively higher risk to preventative and therapeutic strategies to combat breast develop breast cancer than Western women (Harirchi et al., 2000). Therefore, in Iran more advance malignancies needed to be developed.

Anti-differentiation non-coding RNA, (ANCR), is a lncRNA gene located on human chromosome 4 upstream of USP46 gene, and embeds MIR4449 and SNORNA26 within its 1st and 2nd introns. Although these small non-coding RNAs are co-expressed with ANCR, but they are not part of the mature lncRNA transcript. ANCR gene encodes a single 855-nucleotide transcript with diminished expression in different terminally differentiated cell types (keratinocytes, adipocytes and osteoblasts), suggesting that it controls differentiation genes scattered throughout the genome (Kretz et al., 2012; Zhu and Xu, 2013).

In this study, we initially assessed the expression of ANCR in various tumor cell lines, and then examined a potential gene expression alteration of this lncRNA in urinary bladder and breast tumor and non-tumor samples.

Materials and Methods

Cultivation of human cell lines

Cell lines originated from bladder carcinoma (5637), breast adenocarcinoma (MCF-7), hepatocellular carcinoma (HepG2), prostate cancer (PC3), prostatic adenocarcinoma (LNCAP), lung adenocarcinoma (A549), and human colorectal adenocarcinoma (Ht29) were obtained from Pasteur Institute of Iran. They were cultured at 37ºC with 5% humidified CO2 in RPMI or high glucose DMEM medium (Invitrogen, UK) supplemented with 10% FBS (Invitrogen, UK), 100 U/mL penicillin, 100 mg/mL streptomycin, and 25 ng/mL amphotericin B.

Clinical Samples

Urinary bladder tissue samples from cancer patients had been underwent surgery in Labafi-nejad hospitals, were collected whereas breast tissue samples were provided by Iran bank of tumor tissues, in Imam Khomeini hospital. Tissues were immediately snap frozen using nitrogen before extracting their total RNA. In addition, the whole procedure was approved by the ethics committees of Tarbiat Modares University and Labafi-nejad hospital. Patients' written informed consents were also collected prior to sampling. Pathological parameters for tumor grading were evaluated according to the WHO criteria.

RNA Extraction and cDNA synthesis

Total RNA was extracted from both bladder and breast tissues as well as from tumor cell lines, using TRIzol reagent (Invitrogen, USA), according to the manufacturer's instructions. To evaluate the purity and integrity of the isolated RNA, RNA samples were separated by agarose gel electrophoresis and their concentration was measured by optical absorbance at 260 nm. Furthermore, 4 µg of the extracted RNA were treated by RNase-free DNaseI (Takara, Japan) to remove any trace of DNA contamination. Then, 1 µg of DNase-treated RNA was used for Reverse transcription by employing PrimeScript TM Reagent kit (Takara, Japan).

Quantitative Real-time PCR and regular RT-PCR

The specific primers for regular and quantitative RT-PCR to amplify both ANCR (GenBank accession numbers NR_024031.1) and β2 microglubolin (β2M, as an internal control; GenBank accession number NM_004048.2) were designed using Gene runner (version 3.02; Hastings Software), PerlPrimer v1.1.16, and Oligo v 6.54 softwares.

Regular RT-PCR reactions were performed using 10 µl of Taq DNA polymerase master mix RED (Ampliqon, Denmark), 2µl of synthesized cDNA, and 0.5 mM of each primer. PCR cycles were performed using the following conditions: initial denaturation at 94ºC for 5 min, followed by 30 and 25 cycles (for ANCR and β2M amplification, respectively) of 94ºC for 35 seconds, 60ºC for 30 seconds, and 72ºC for 35 seconds, with a final extension at 72ºC for 10 minutes. The aforementioned primers amplified a 472 bp fragment of ANCR and a 167 bp fragment of β2M. Then, the PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light. The validity of PCR products was confirmed by direct DNA sequencing (Microgen, South Korea). The sequences of the forward and reverse primers were shown in table 1.

2 µl of cDNA, 10 µl of SYBR-Green ready mix, and 0.1 µl of Rox, and 0.5 µM of each specific primer were used to carry out quantitative PCR (q-PCR). β2M gene was used as an internal control, and the expression of other genes were normalized to its expression level.

Table 1. Primer Sequences

Primer name	Sequence
β2MF	5-GGGTTTCATCCATCCGACATTG-3
β2MR	5-TGGTTCACACGGCAGGCATAC-3
ANCR-F1	5-GCCACTATGTAGCGGGTTTC-3
ANCR-R1	5-CAGAGTATTCAGGGTAAGGGTC-3
ANCR-R2	5-GTAGTTGTCAACCTGCGCTAAG-3

ABI 7500 real-time PCR system (applied Biosystems, Foster city, CA) was used to perform the PCR using the following cycling conditions: initiation at 95°C for 15 minutes, amplification for 40 cycles with: denaturation at 95°C for 15 seconds, annealing at 62.5°C for 30 seconds, and extension at 72°C for 30 seconds. To validate the PCR products, melt curves were analyzed, and amplified products were sequenced**.**

Statistical Analysis

The relative expression values were calculated by using the ΔΔCT method, by normalization to the internal control β2M, and those of non-tumor samples in both breast and bladder tissues, both used as calibrators to calculate the fold change of relative expression. The significance of difference in the gene expression levels was determined by student's t-test using GraphPad Prism 6.0. Furthermore, all qPCR experiments were carried out in duplicates**.**

Results

Two sets of primers were designed to delineate the expression patterns of ANCR in various cell lines, urinary bladder tumors, and breast tumors (Figure 1). The designed primers serve to amplify a 472 bp (using F1 and R1 primers) and a 171 bp (F1 and R2 primers) fragment of ANCR cDNA by regular RT-PCR or quantitative RT-PCR, respectively. The authenticity of the PCR products was further confirmed by DNA sequencing. Moreover, DNase treatments as well as no-RT reactions, as negative controls, were employed to avoid genomic's DNA amplification.

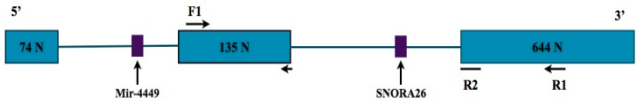

Figure 1. Genomic organization of ANCR. Two pairs of primers were designed; F1/R1 primers employed in regular RT-PCR and F1/ R2 primers used in qRT-PCR.

ANCR expression was delineated in seven different cell lines (5637, MCF7, HEPG2, PC3, A549, and Ht-29), and all of the examined cell lines were found highly expressing ANCR (Figure 2).

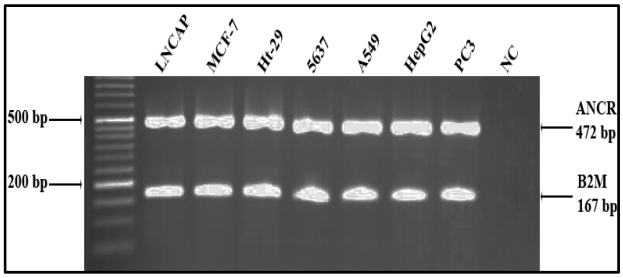

Figure 2. : Expression of ANCR in 7 different cancer cell lines. ANCR expression was detected in LNCAP, MCF-7, Ht-29, 5637, A549, HepG2, and PC3 cell lines. B2M gene was used as an endogenous control, and No-RT control (NC) sample was employed to validate the accuracy of RT-PCR products.

Moreover, the primers for conventional RT-PCR were designed in a way to permits the detection of any possible novel variants of ANCR. However, it failed to recognize and alternative spliced form for ANCR.

Additionally, the expression profile of ANCR was analyzed in 10 pairs of breast tumor and non-tumor samples, as well as 10 pairs of urinary bladder tumor/non-tumor surgical specimens. Initially, ANCR expression was normalized to that of endogenous control, β2M. Moreover, the non-tumor samples were used as calibrators to calculate the relative expression of ANCR. Based on our real-time data, ANCR found to have a higher expression level (p = 0.003; Figure 3A) in tumor samples, compared to that of apparently normal tissues obtained from the margin of same patients. Similar observation was made on upregulation of ANCR in bladder tumor samples, compared to their paired non-tumor specimens. However, the latter overexpression was not statistically significant (p = 0.3; Figure 3B).

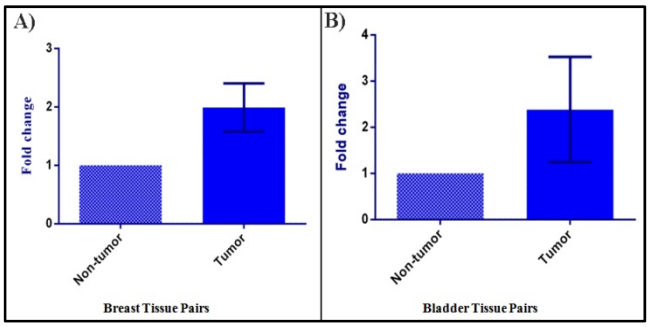

Figure 3: ANCR expression in breast and bladder tumor and non-tumor tissue pairs. A) A significant (p = 0.003) upregulation in the expression of ANCR was detected in breast tumor specimens, in comparison to the non-tumor marginal samples obtained from the same patients. B) Similarly, in bladder samples ANCR was upregulated in tumor samples, but such alteration was not statistically significant ($p > 0.05$).

Discussion

In recent years, numerous reports have identified several lncRNAs with important regulatory parts in initiation and progression of cancers (He et al., 2014; Huarte and Rinn, 2010; Shahryari et al., 2014; Tian et al., 2014; Xiang et al., 2014). Such non-coding RNAs play crucial roles in almost every aspect of cell biology, from epigenetics modification to posttranscriptional regulation and splicing (Ip and Nakagawa, 2012; Mercer et al., 2009; Soreq et al., 2014; Yang et al., 2011; Zhou et al., 2014). These processes are linked directly to the genetics of cancer initiation and development. Therefore, aberrant expression of lncRNAs is expected to be a causative event of tumorigenesis in different tissues.

In the light of available statistics, both breast and urinary bladder cancers are associated with high incidence and mortality rates, therefore finding novel diagnostic and prognostic biomarkers, as well as to develop more efficient therapeutic strategies are needed. Accordingly, several researches have recently focused on misregulation of lncRNAs in bladder and breast cancers, and proposed such transcripts as potential diagnostic, prognostic, and therapeutic biomarkers (Crea et al., 2014; Lin et al., 2014; Shahryari et al., 2014; Zhang and Leung, 2014).

Considering the reports linking perturbations of lncRNAs and cancer, we aimed here to explore a possible link between ANCR expression and tumorigenesis in both breast and urinary bladder tumors. ANCR was reported to be downregulated in terminally differentiated keratinocytes and osteoblasts (Kretz et al., 2012; Zhu and Xu, 2013). These findings raised the possibility that this transcript has a critical function in the transition from progenitor to differentiated state of the cell. To the best of our knowledge, this is the first report to delineate the expression of ANCR in breast cancer and UBC.

Similar to HOTAIR, a lncRNA that interacts with Polycomb Repressive Complex 2 (PRC2) to regulate the chromatin state and found to be overexpressed in breast cancer (Gupta et al., 2010; Wu et al., 2013), our data showed that ANCR was also significantly upregulated in breast tumor tissues in comparison with the non-tumor control samples. On the other hand, in bladder cancers, Urothelial Cancer Associated-1 (UCA1) is highly expressed in embryonic tissues, bladder and other cancers, but not in adult tissues or adjacent non-tumor tissues, indicating that UCA1 may be involved in both embryonic development and tumorigenesis (Wang et al., 2008). Likewise, ANCR showed an overexpression in the urinary bladder tumor samples, but such increase was not statistically significant, probably due to a low number (10 pairs) of samples. These findings may highlight a strong correlation between the aberration in ANCR expression and tumorigenesis. Nevertheless, these data are preliminary and need to be validated by using more tissue samples. Therefore, with employing more samples, subdivided in different grades and stages of malignancies, we could better analyze the suitability of ANCR as a potential diagnostic and prognostic biomarker.

In this study, we also delineated the expression of ANCR in different cell lines originating from various tissues (5637, MCF-7, HepG2, PC3, A549 and Ht-29). The fact that ANCR is highly expressed in these cell lines suggests that it has probably a general causative role in other types of cancers.

In Conclusion, we depicted the expression pattern of ANCR in both urinary bladder and breast tumors. Our data is the first report on distinct expression of ANCR in breast and bladder cancers, demonstrating a possible link between the expression level of ANCR and tumorigenesis. Moreover, with further validation, ANCR can potentially be considered as a novel tumor biomarker with potential diagnostic, prognostic and therapeutic value.

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