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Mutational Screening of the Negative Regulatory Region of Astrocyte Elevated Gene -1 Promoter in Hepatocellular Carcinoma Patients

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Objective: This study is aimed to assess the correlation between negative regulatory region of AEG-1 promoter and genetic susceptibility to hepatocellular carcinoma in Iranian population.
Methods: The negative regulatory region of human *AEG-1* promoter was explored in 50 Iranian HCC patients. The PCR-sequencing method was used for investigating *AEG-1* promoter polymorphisms.
Results: This analysis did not reveal the presence of any deletions, insertions, or mutations in this region of AEG-1 promoter.
Conclusion: Mutations in the negative regulatory region of AEG-1 promoter have no significant relevance with hepatocellular carcinoma. Nevertheless, to our knowledge,

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this is the first study to investigate the mutations of the negative regulatory region of AEG-1 promoter in HCC patient. The role of AEG-1 promoter played in HCC needs to be further studied.

Keywords: Astrocyte elevated gene -1; promoter; hepatocellular carcinoma; mutation.

ABBREVIATIONS

HCC : hepatocellular carcinoma
AEG-1 : Astrocyte elevated gene 1
PHFA : primary human fetal astrocytes
TBE : Tris- base- EDTA
PCR : Polymerase Chain Reaction.

1. INTRODUCTION

Approximately 50,000 new cases of cancer occur each year in the Iranian population of 70.4 million [1]. Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world and is estimated to cause approximately half a million deaths annually [2], with a high incidence in some countries, especially in the Asian continent [3].

HCC is a tumor with rapid growth and early vascular invasion [4]. It is also highly resistant to standard chemotherapy [4]. Major risk factors for hepatocellular carcinoma include infection with HBV or HCV, alcoholic liver disease, and most probably nonalcoholic fatty liver disease [5]. The treatment options for HCC depend on the stages and grades of the disease [6]. Surgical resection, radiofrequency ablation, and liver transplantations are the treatments of choice with localized disease [6].

Astrocyte elevated gene 1 (AEG-1), a novel oncoprotein, has been implicated in oncogenesis and cancer progression in various types of human cancers [7]. Human AEG-1 gene is located in Chromosome 8q22 having 12 exons/11 introns. Chromosome 8q22 is known to be a hot spot for genomic alterations in several cancerous cells involving HCC and breast cancer [8-10]. AEG-1 was cloned by rapid subtraction hybridization as a gene induced in primary human fetal astrocytes (PHFA) infected with HIV-1 or treated with TNF-α [11]. General localization of AEG-1 is in the cytoplasm and also detected in the ER/nuclear envelope [12, 13].

Expression of AEG-1 was extremely low in human hepatocytes, but its levels were significantly increased in human HCC [4]. Over expression and inhibition studies, in both in vitro and in vivo models, reveal the importance of AEG-1 in regulating multiple physiologically and pathologically relevant processes, including proliferation, invasion, metastasis, and gene expression [14].

As reported by Lee and colleagues in 2006, the human *AEG-1* promoter has positive (-459/-302) and negative (-738/-460) regulatory regions (Fig. 1). The negative regulatory regions have putative RAR-α and YY1 binding sites, which act predominantly as repressors of transcription [15].

As it was mentioned before, AEG-1 is over expressed in more than 90% of HCC patients and plays a significant role in mediating aggressive progression of HCC [6]. Thus, we predict an important role in gene suppression for AEG-1 negative promoter region, with putative RAR-α and YY1 binding sites, and believe its variants may be responsible for the over expression of AEG-1 gene in HCC patients.

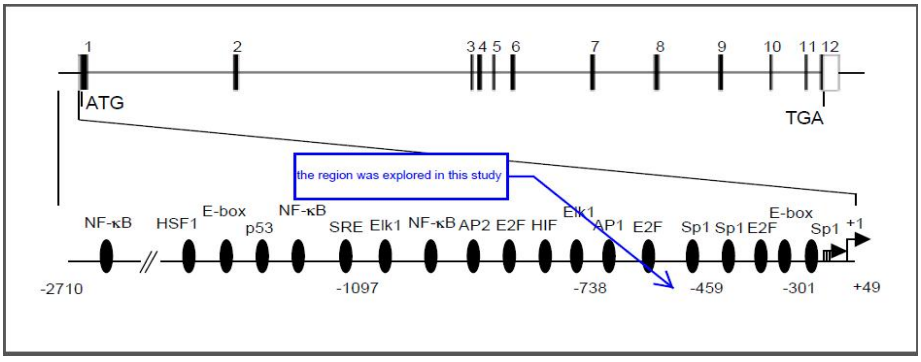

Fig. 1. Schematic diagram of AEG-1 promoter (13)

This study was aimed to investigate the association between the *AEG-1*negative promoter regions variants in hepatocellular carcinoma patients of Iranian context, in which might be a reason for the increased expression of this gene. To our knowledge, this region has not been previously tested in the hepatocellular carcinoma patients.

2. MATERIALS AND METHODS

2.1 Sampling

The studied population comprised 50 HCC patients. The patients were recruited from the Mazandaran, Tehran, Shiraz, Esfahan, Sistan and Balochestan and Gorgan Provinces in Iran (Table 1). HCC patient diagnosis was based on the standard criteria (pathologic, histopathology and demographic). In this study, the average age of the selected hepatocellular carcinoma patients was 59±2 years, in which consisted of 47% female and 53% male genders. Written informed consent was obtained for each subject.

Table 1. HCC sample distribution according to the regions of origin

Regions	Number of samples
Tehran	10
Mazandaran	9
Shiraz	9
Esfahan	8
Sistan and Balochestan	8
Gorgan	6
Total	**50**

2.2 Deparaffinization

Paraffin-embedded tissue sections were deparaffinized with 100% xylene for three times. To remove the residual xylene, the samples were washed five times with ethanol. The sections were then left in a 40 ºC oven to dry the tissues. After tissues were dried, 500 microliter lyses solution was added to each samples (40 mMTris, 1mM EDTA, 0.5% Tween-20, 0.5 µg/µl proteinase k, pH=8) [16,17].

2.3 DNA Extraction

Genomic DNA was extracted from tissues by using the phenol/chloroform procedure [15]. DNA concentration and the purity of each sample were measured by Picodrop. DNA samples were routinely stored at -20 ºC.

2.4 Sequencing Analysis

The negative regulatory regionof*AEG-1* promoter (accession number: AF411226) was sequenced to investigate genetic variants in DNA samples obtained from the 50 cases. The distribution of promoter variants were determined by Polymerase Chain Reaction (PCR) - sequencing method. Briefly, the region was amplified with the following primers:

For ward 5'- CCACCAGGGAGAAAAAGCG -3'

Reverse 5'- GAAGGCGTCCACCAATTAAC -3'.

Most human gene promoters contain G+C sequences that are very difficult to amplify. In this study, we propose an improved PCR method to be used for successful amplification of the *AEG-1* gene promoter region that exhibit >70% G+C content in a sequence of approximately 238 bp, using touchdown-PCR and co-activator Dimethyl sulfoxide (DMSO) [18].

The touchdown-PCR was carried out on Corbett Thermal Cycler using the following conditions: an initial denaturation at 96ºC for 7 min, followed by 16 cycles of 94ºC for 1 min, 60ºC (0.5ºC decrease per cycle) for 45sec, 72ºC for 1 min, then another 15 cycles of 94ºC for 1min, 52ºC for 45 sec, 72ºC for 1 min, and a final extension step at 72ºC for 7 min. The PCR products were separated on 2% agarose gels in Tris- base-EDTA (TBE) buffer stained with DNA-safe and visualized by Gel Doc Imaging System. All sequencing data was produced by the Genomic Analysis Facility (Puia Gostar Gene, Tehran, Iran) Chromas software [18].

3. RESULTS

Samples were received from different provinces of Iran and their DNA were extracted. Comparative analysis between the ratio of absorbance at wavelengths of 260 and 280 nm was performed. Average ratios obtained in the range of 1.7 were suitable for DNA.

To ensure more, it was followed by electrophoresis (Fig. 2). The AEG-1 gene promoter was amplified by touchdown PCR. All samples had a clear runway suitable for sequencing (no non-specific bands and primer dimer, respectively). Samples were electrophoresed in front of 100-bp size marker (Fig. 3).

Data obtained from sequencing were analyzed (Fig. 4). Sequences acquired for genotyping were then compared with the standard sequence (Fig. 5). Finally, we did not find any diagnostic alterations in the negative regulatory region of AEG-1, no mutation, insertion or deletion in HCC patient.

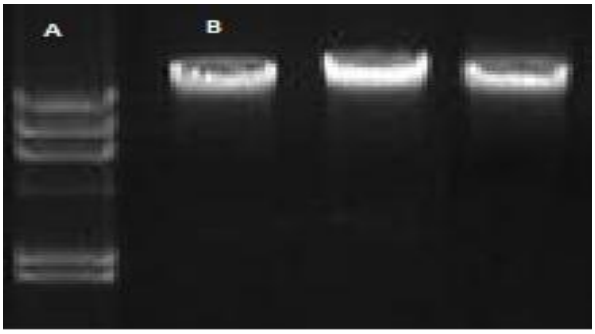

Fig. 2. Genomic DNA were analyzed on 1% agarose gel. A: Marker, 1Kb DNA step ladder marker, B: Genomic DNA isolated from HCC tissues

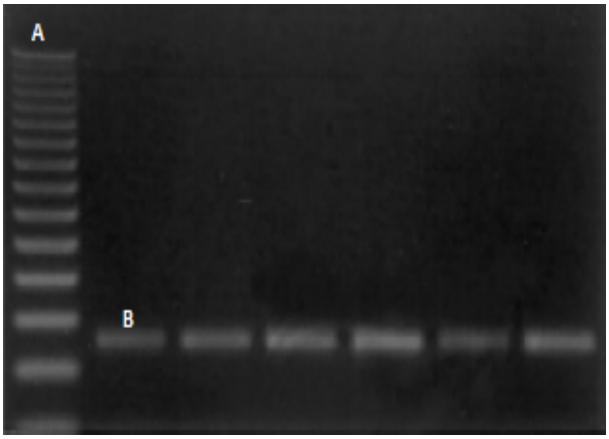

Fig. 3. Agarose gel electrophorsis. A:Marker, 100 bp DNA step ladder marker, B: PCR product of AEG-1 promoter

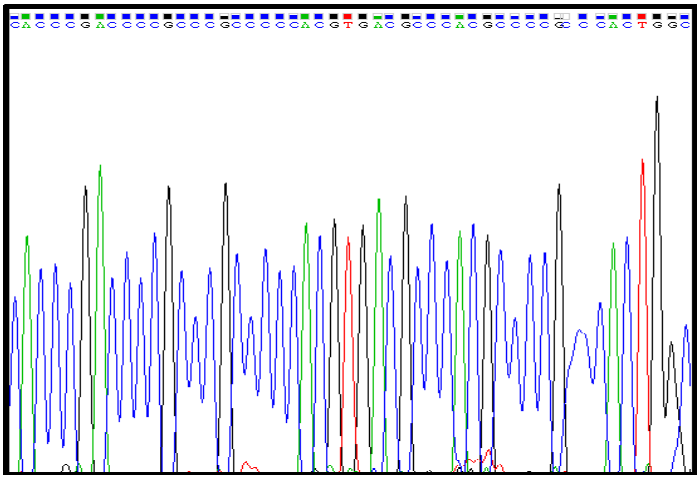

Fig. 4. AEG-1 promoter chromatogram

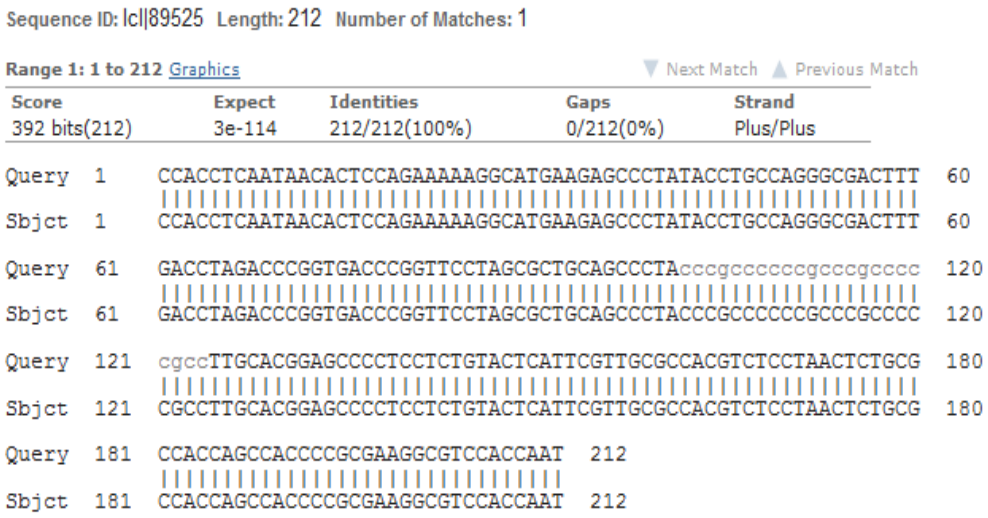

Fig. 5. BLAST result of AEG-1 promoter sequence with reference sequence

4. DISCUSSION

Based on the in vitro and in vivo studies in animal models and expression analysis using patient samples, it is eminently clear that AEG-1 plays a decisive role in the process of tumorogenes is in multiple organs [14]. Based on this information and the essential role of the promoter in gene expression, negative regulatory region of AEG-1 promoter that is consisted of putative RAR-α and YY1 binding sites was chosen for mutation analysis. In another study conducted by our team, positive promoter regulatory regions of AEG-1 were explored. Sequencing data showed genetic variations in 11 HCC patients and 3 healthy controls. Among them, one novel SNP was found in the binding site of Activator protein-2 (AP2) transcription factor (-483 A to C), which had an association with susceptibility to HCC (P =0.05); however, no association was found for other observed variations [19].

The current analysis did not reveal the presence of any mutation or insertion- deletion in this region of AEG-1 promoter. The lack of variants in AEG-1 promoter noted in this study and the absence of transcriptional silencing rules out the hypothesis concerning the role for the gene variants in HCC susceptibility. This is in agreement with results of a similar study in which AEG-1 promoter upstream of the start codon was screened for mutations in colon cancer collected from the Chinese population [20]. These data suggest that the promoter mutations located in this region of AEG-1 are not responsible for HCC susceptibility, and thus argues against their possible role in HCC development. Over expression of the AEG-1 in HCC patient may be accompanied by some other reason such as: A) another regional mutation of AEG-1 gene sequence, e.g. positive regulatory regions in promoter, exons and introns, B) over expression of transcription factors, that have a binding site in AEG-1 promoter, C) alternation of signaling pathway that are related to HCC progression. Second, it may simply be that there is very little relationship between the AEG-1 genetic variants and the risk of HCC.

5. CONCLUSION

Our understanding of the functions of the AEG-1gene negative promoter region is still very limited, and its exact role in AEG-1 over expression remains unknown. Further studies will be required to determine the role of this region, if any, in the expression of AEG-1 gene in HCC tumor. Since AEG-1 has been demonstrated as a key regulator in the complex network of oncogenic pathways, additional investigations are needed to further clarify the functional role of this gene and its association with HCC development and recurrence. While additional studies on the AEG-1 gene is necessary, variants of AEG-1 may prove to be potential markers for HCC development, prognostic indicators for disease progression, and possible foci for future targeted therapy.

ETHICAL APPROVAL

The ethical issues of this project were approved by the ethics committee of Golestan University of Medical Sciences. Ethical issues including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc. have been completely observed by the authors.

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DISCLOSURE OF BENEFITS

The authors have not disclosed any affiliation or financial involvement with organizations or entities with a direct financial interest in the subject matter or materials discussed in the manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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